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PROGRESS REPORT

INVESTIGATION OF PEROGNATHUS AS AN EXPERIMENTAL ORGANISM FOR RESEARCH IN SPACE BIOLOGY

A SUMMARY OF PROGRESS
1 October 1963 through 31 December 1964

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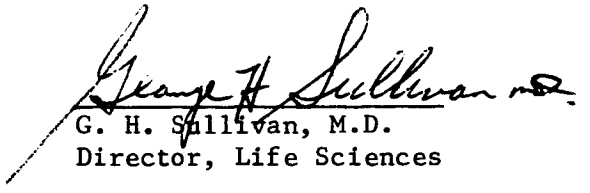

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A SEARCH FOR MECHANISMS OF RADIATION RESISTANCE IN POCKET MICE

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INTRODUCTION

The report that members of the genus Perognathus withstand whole body radiation doses up to 1400 rads without appreciable mortality represented the first of several suggesting high natural radiation resistance scattered among species of wild rodents (6,3,9). Several species of Peromyscus, Sigmodon hispidus, Reithrodontomys humulis and the Mongolian gerbil, Meriones unguiculatus, are among those now listed as exhibiting a degree of radiation resistance approaching that of Perognathus.

Without belaboring the definition of the term "radiation resistance" we pose the question: How is it possible for these animals to withstand radiation doses twice that tolerated by other mammals, including closely related rodents?

The series of brief experiments reported here was designed to study this question in Perognathus (6,7,8).

I. THE OXYGEN EFFECT

The presence of oxygen in a living system during exposure normally enhances the damaging effect of ionizing radiation. Conversely, removing oxygen from the system - e.g., producing hypoxia in an animal - protects against radiation damage.

If Perognathus is radiation resistant by virtue of some ability to make vital cells hypoxic during exposure, this hypoxia might be abrogated by forcing oxygen into the tissues. In this experiment animals were administered midlethal* irradiation while breathing 100% oxygen at 3 atmospheres pressure. It was hypothesized that survival of any of these animals would argue strongly against a physiological hypoxia mechanism.

It was further postulated that if severe visceral hypoxia occurs in these animals, for example, as a result of stress response to handling during the pre-radiation period, protection would be afforded against both hemopoietic and gastrointestinal death. In this instance, splenic involvement could be tested by simply removing it prior to radiation. If the spleen does provide the necessary hemopoietic "seed" tissue for the bone marrow in intact post-irradiation Perognathus, its absence should prove fatal to the splenectomized Perognathus. Survival of any splenectomized pocket mice following midlethal irradiation would argue against a visceral hypoxic mechanism.

Materials and Methods

Six groups of 10-12 animals each were established from our main colony as described elsewhere (6). One group of 10 animals received 1400 rads total body Co^{60} radiation** while maintained at 3 atmospheres pressure in 100%

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* An exposure that results in some but not 100% acute death.
** Radiation factors described elsewhere (6).

oxygen. The animals were maintained under these conditions for 30 minutes prior to the irradiation to insure tissue saturation. A control group of 10 animals received the same treatment except for the irradiation.

One group of 10 animals was splenectomized 5 to 10 days prior to the irradiation date. Nine survivors of this group were administered 1400 rads irradiation.

One group of 12 animals served as irradiation controls, receiving 1400 rads total body irradiation but no other treatment. A final group of 10 animals served as handling controls.

The 1400 rads dose administered to all irradiated animals in these experiments was chosen because in P. longimembris it results in some 30-day deaths, but 30-day survival is usually between 60% and 90%. Rarely do deaths occur after the acute period is passed; however, long term survivors show certain late effects.

Modes of radiation death in P. longimembris are categorized under three headings based on gross autopsy findings. These are gastrointestinal, hemopoietic and respiratory death. Since micropathology was not performed, they are considered probable causes of death.

In these studies, gastrointestinal death is characterized by: (1) soil plug in perianal region, (2) intestine containing yellowish mucous or bloody fluid, (3) stomach empty or containing mucous only, (4) rapid deterioration of viscera following death.

Hemopoietic death is characterized by: (1) periorbital bleeding, (2) general or local petechaiae - generally axillary and neck region, sometimes stomach and intestine, sometimes brain, (3) massive intracranial hemorrhages, (4) massive hemorrhages in other regions of body.

Respiratory death is characterized by: (1) bloody crusts around nares and mouth, (2) bloody fluid in pleural cavity, (3) large portions of lungs hemorrhagic, consolidated or hepatized.

II. METABOLIC RATE EFFECT

It is well known that post-irradiation periods of reduced metabolic rate slows radiation damage and delays death in lethally irradiated mammals. On the other hand, lowered metabolic rate as it occurs in hibernation postpones, but does not enhance, recovery (23). In fact, deteriorization continues at a slow rate in the post-irradiation hibernating hibernator. This fact is demonstrated by contraction of the period of acute death following arousal from hibernation as compared to the same period without intervening hibernation. Since Perognathus normally undergoes cyclic periods of metabolic depression (and these appear to be more severe following irradiation) (1,14), the question of whether merely protracting the acute death period is responsible for high $LD_{50/30}$ values arises. To test this possibility a number of animals were forced to maintain their body temperature during the 30-day period immediately following total body midlethal irradiation.

Materials and Methods

Immediately after receiving 1400 rads total body irradiation as described previously, 8 P. longimembris were placed in a multi-channel, continous recording respirometer (6,14). The ambient temperature was maintained at 35°C. This value is the lower value of a range of temperatures of a normally active Perognathus maintained at room temperature. The mice remained in the chambers for the entire 30-day post-irradiation period, except for one brief period of cleaning each week. Oxygen consumption was recorded automatically during the entire period.

A control group was subject to the same conditions, but for 6 days rather than 30 days.

Results

Survival of splenectomized, oxygen treated, and normal animals over the 30-day period immediately following 1400 rads total body Co^{60} irradiation is shown in Table 1. Four of 9 splenectomized, and 6 of 9 oxygen animals survived the dose and were alive at 30 days post-irradiation. During the same period, 4 of 12 normal animals which received the 1400 rad dose died. No deaths occurred in the non-irradiated control groups.

Deaths in the splenectomized and untreated irradiated group followed the normal time course for pocket mice. No deaths occurred prior to 8 days post-irradiation and few after the third week. Deaths in the oxygen-irradiation group occurred in the fourth week. Most of the animals that survived the acute period are still alive approximately one year later.

Table 1

Survival of pocket mice following 1400 rads total body irradiation while in 100% oxygen at 3 atmospheres or following recovery from splenectomy

Group	Initial Number	Treatment	Dose (rads)	30-day Survival
1	9	100% O_2, 3 Atmos.	1400	6
2	10	100% O_2, 3 Atmos.	0	10
3	10	Normal Oxygen	0	10
4	12	Normal Oxygen	1400	8
5	9	Splenectomized	1400	4

Results

Three of the 7* initial animals survived the radiation and the 30-day period following. All of the deaths occurred between the 8th and 12th days.

A summary of metabolic rate values for normal and 1400 rads irradiated animals subjected to continuous 35°C ambient temperature appears in Table 2. The average high metabolic rate of normal mice was lower than that of irradiated mice.

Table 2

Mean oxygen consumption of normal and irradiated *Perognathus longimembris* maintained at 35°C during period immediately following exposure

Treatment	Number of Animals	Oxygen Consumption - Average Values		
		Mean High	Mean Low	Mean 24 hr. Period
NONIRRADIATED 6 Day Period (Values for days 1-6)	7	4.45 ± 0.23**	1.36 ± 0.07	2.12 ± 0.05
IRRADIATED (1400 rads) Surviving 30-Day Period (Values for days 1-9)	3	5.19 ± 0.29	1.31 ± 0.16	2.07 ± 0.23
Dying Within 9-Day Period (Values for days 1-8)	4	5.39 ± 0.67	1.35 ± 0.14	2.28 ± 0.07

* Initial group of 8 reduced to 7 due to one death by drowning.
** Mean ± S.D. ml/gram-hour STP.

III. COMBINED EFFECT OF RADIATION AND HYPOMETABOLISM

To test the possibility that fortuitous transient reduction of body temperature during exposure might enhance post-irradiation survival in Perognathus, a group was irradiated while torpid. Immediately following irradiation the animals were returned to room temperature and maintained under normal conditions until their death.

Materials and Methods

Four groups of 12 male animals each were segregated from our main colony. The animals were collected in Nevada during the Spring of 1963. They were approximately 1 year old with a mean weight of 9.1 grams.

Food was withheld from all groups for 24 hours prior to radiation. In the afternoon of the day before irradiation, all 4 groups were placed in plastic compartmented boxes. Two of the groups were then placed inside a cooling unit set up in the radiation source room where they remained overnight. The other 2 groups were kept at room temperature ($22^{\circ}C$).

Within one hour after the animals were placed in it, the temperature within the cooling unit was reduced from room temperature to $9^{\circ}C$. The temperature remained at $9^{\circ}C$ for the duration of the experiment.

One group maintained torpid at $9^{\circ}C$ and one group maintained active at room temperature ($22^{\circ}C$) were simultaneously administered 1700 rads Co^{60} radiation at a dose rate of 36.2 r/min. The other 2 groups were held as hypothermia and normal controls. The total time of exposure was 48 minutes following which time all of the animals were returned to their individual cages and food restored.

The hypothermic animals were observed just prior to, and following irradiation. Prior to irradiation they all were deeply torpid. One

animal showed signs of awakening when the lid of the cooler was removed just after irradiation. It took approximately 1 hour for all of the animals which were made hypothermic to return to a normal active state after being returned to room temperature.

The animals were observed daily during the first week following irradiation. As the first animals began to die on the 8th day, they were observed several times a day. Autopsies were performed as soon as possible after death.

Results

Table 3 shows the mortality pattern in the two irradiated groups (1700 rads normometabolic and 1700 rads hypometabolic) and compares these with mortality in other Perognathus longimembris radiation experiments reported previously (6). Mean survival time was the same for both groups - hypometabolic 10.1 days, normometabolic 10.8 days. No controls died during the same time period.

Although there is no difference in survival between normometabolic and hypometabolic pocket mice administered 1700 rads whole body irradiation, the hypometabolic state produces a somewhat more uniform death rate. No significance can be attached to this observation in such small groups; however, comparison with mortality patterns of several radiated normometabolic groups seems to corroborate this point (Table 3).

Gastro-intestinal hemorrhages were seen at autopsy in all but one of the irradiated animals. In that one, early death was attributed to respiratory failure possibly due to an infection antedating the radiation. In several instances both severe gastrointestinal damage and respiratory disease were noted at autopsy. In addition, as noted on other occasions in post-irradiation necropsy of pocket mice, massive intracranial hemorrhages were seen in 4 of the 24 irradiated mice.

Whole-Body Co^{60} Dose (Rads)	Init. Num. of Mice	Number Dead Each Day	Number Surviving
2000 Normometabolic	25	2 3 1 4 3 1 3 1 5	2
1800 Normometabolic	25	2 1 2 3 4 1 4 3 2 2 1	0
1700 Normometabolic	12	1 2 4 1 2 1 1	0
1700 Hypometabolic	12	5 3 3 1	0
1600 Normometabolic	25	1 1 1 2 3 2 1 3 2 3 1	5
1400 Normometabolic	12	1 1 1 1	8
1400 Normometabolic	48	2 1 1 4 3 1 2 1 1 3	29
1300 Normometabolic	25	1 1	23

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Days Post-Irradiation

TABLE 3. Comparison of 30-Day Survival of Pocket Mice Irradiated At Several Dose Levels While Normometabolic or Hypometabolic.

IV. IRRADIATION DURING HYPOXIA

Since artifically-induced tissue hypoxia is a known means of producing radiation resistance in mammals, a group of Perognathus longimembris were irradiated while they were in severe hypoxic hypoxia. Survival of irradiated hypoxic mice was compared to survival of mice administered the same dose while in a normal oxygen environment.

Materials and Methods

Sixty adult Perognathus longimembris were segregated from our holding colony and randomly divided into 3 groups of 20 each. One group received 2100 rads total body irradiation; the second group received 2100 rads total body radiation while hypoxic; and the third group received hypoxia only.

Pocket mice proved to be extremely tolerant to low ambient oxygen. They appear to be even more tolerant than other hibernators (2). In order to produce hypoxia in these animals, ambient oxygen had to be reduced below 5% in slow increments. This was accomplished by reducing oxygen in a nitrogen-oxygen breathing mixture piped into a lucite exposure chamber.* The degree of hypoxia was judged by clinical signs of stress and by survival studies in control groups. In preliminary studies it was determined that by reducing oxygen in increments, 75% of a normal population of pocket mice would survive 25 to 30 minutes in 2.6% oxygen at room temperature.

This oxygen concentration was judged sufficient to insure complete hypoxia in all of the mice. A radiation dose rate was selected to obtain, within the 25 to 30 minutes time period, delivery of a total dose sufficient to produce 100% acute (< 30-day) deaths in unprotected pocket mice.

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* Details of method in report to NASA, NSL 64-29-3, April - June 1964.

Irradiation was delivered from a 5000 curie Co^{60} source at a dose rate of 105 r/min. The total dose (2100 rads) was delivered in about 20 minutes. Both ferrous sulphate and phosphate glass dosimeters were used.

The group irradiated under hypoxia was returned to a normal oxygen atmosphere immediately after irradiation. The total period in 2.6% oxygen was less than 25 minutes. The hypoxia control group had identical treatment except for the irradiation. The group irradiated without hypoxia was handled similarly and administered the same dose under the same conditions as the irradiation-hypoxia group except, of course, for the hypoxic atmosphere.

After irradiation all groups were returned to the holding facility and maintained as described earlier. They were checked twice daily, in the morning and in late afternoon, and observed for signs of radiation sickness and deaths. Dead animals were autopsied to determine gross pathology.

Results

Survival of mice in this experiment is shown in Table 4. At 30 days post-irradiation all of the animals that survived the exposure period were alive in the hypoxia groups and all of the animals irradiated without hypoxia were dead.

Mortality in the group that received 2100 rads without hypoxia occurred in the 8th through the 15th day, with a mean survival time of 11 days. The most common autopsy finding in this group was large amounts of bloody fluid in the small intestine. In addition, respiratory infection characterized by large foci of consolidation or hepatization was seen in 7 of the 20 dead mice. Intracranial hemorrhages were seen in 4 animals.

TABLE 4

Survival of pocket mice receiving 2100 rads total body irradiation with and without hypoxia

Group	Initial Number	Treatment	Number Surviving Exposure Period	Number Surviving at 30 days
A	20	Irradiation + Hypoxia	8*	8
B	20	Irradiation only	20	0
C	20	Hypoxia only	14	14

All of the animals that survived hypoxia, both irradiated and controls, are alive and appear healthy at the time of this writing (8 months post-irradiation). During the period in which the unprotected irradiated mice were dying, one of the irradiated-hypoxia animals appeared lethargic for about 1 day. Aside from this single observation, hypoxia alone or hypoxia and irradiation did not affect the survivors as judged by outward signs.

*NOTE: It is difficult to account for the high mortality during irradiation in the group receiving simultaneous irradiation and hypoxia. It is possible that the combined stresses were too severe, or perhaps our preliminary data on hypoxia tolerance was insufficient. There is also a possibility that occlusion of the air line may have occurred briefly during the acclimitization period. In spite of the severe mortality during irradiation, the fact that all 8 survivors are still alive while all of the unprotected mice died before the 16th day post-irradiation, indicates that the hypoxia treatment was successful.

V. RESPONSE TO MASSIVE GAMMA IRRADIATION

Pocket mice were administered massive doses of gamma-irradiation to determine whether they follow the pattern of CNS death familiar in other mammals.

Materials and Methods

Four groups of 10 adult P. longimembris each were established. Three of these groups were administered radiation doses ranging from 8.5 to 24.1 krads. One group was kept as controls.

In order to obtain massive irradiation, the animals were lowered into a 5,000 curie Co^{60} source composed of 12 eight inch long Co^{60} needles arranged to form a cylindrical basket twelve inches in diameter. The dose rate within the source is 5.2 krads per minute. Because the mechanism for raising and lowering samples into the source is slow, a transit dose of 8.5 krads is accumulated. Total dose is determined by adding dose received while within the source to transit dose.

Of the three groups exposed, one received the transit dose of 8.5 krads only. A second group received the transit dose plus one minute exposure within the Co^{60} source accumulating a total of 13.7 krads. The third group was administered 24.1 krads during a 3 minute exposure within the Co^{60} source.

Results

The animals administered 8.5 krads exhibited no locomotor or other behavioral effects either immediately post-irradiation or any time following until death. Mean survival time in this group was 7.8 days with all of the deaths occurring between the 5th and 10th days.

Animals administered 13.7 krads and 24.1 krads all showed early locomotor disturbances. Ataxia was noticed as soon as the animals were replaced into their individual cages from the exposure cages. Some showed extreme sensitivity to handling or other stimuli. For example, they reacted violently to prodding with a pencil. Others showed uncommon aggressive behavior in the first six hours after irradiation. Behavioral effects were most pronounced in the highest dose group (24.1 krads). This group had a mean survival time of about one day. All of the animals died before 21 hours post-irradiation, except one which survived an additional 24 hours.

Mean survival time in the group receiving 13.7 krads was 7.9 days. Eight of the ten died between the 7th and the 11th day. Two died earlier; one on the 1st day and the other on the 4th day post-irradiation.

No controls died during the course of this experiment.

Autopsies were performed on all animals. There were no grossly observable pathological conditions in the 24.1 krad group. The brain and vital organs of thoracic and abdominal cavity of these animals all appeared normal upon gross examination.

All animals that died between the 5th and 11th days showed signs of severe gastrointestinal bleeding. In most instances the small intestine was full of bloody fluid. There were no signs in any of these animals of cranial hemorrhages as is often seen in pocket mice administered 1500 to 2000 rads.

A dose-survival curve based on these data and data from preceding reports is shown in Figure 1.

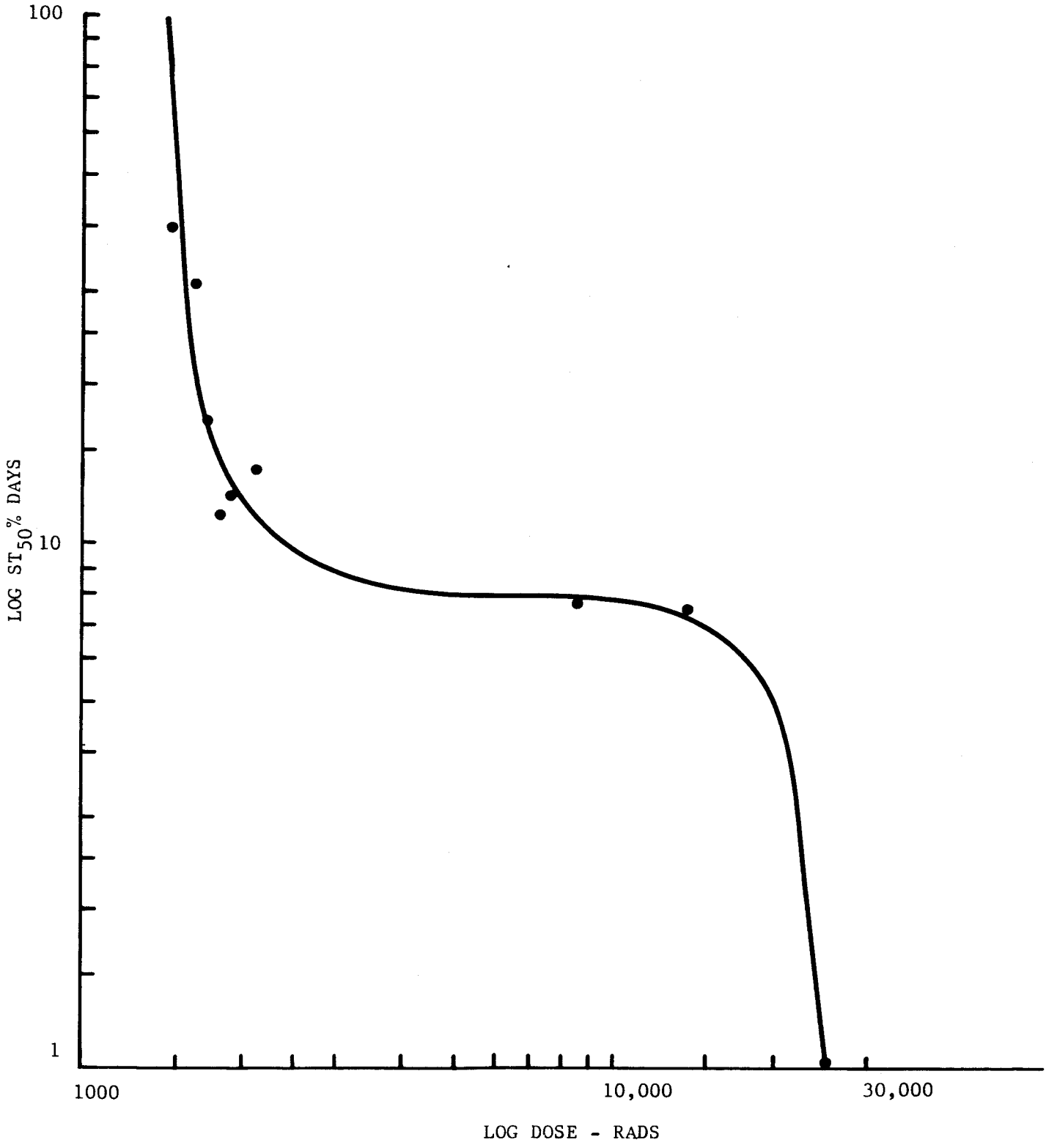

FIGURE 1. Survival (ST_{50}%) of pocket mice after various doses of gamma irradiation (curve visually fitted)

DISCUSSION AND CONCLUSIONS

Survival or death in irradiated multicellular animals is dependent upon the ability of a critical number of cells in systems vital to the integrity of the organism to survive and to repair injury. Variation in response of cells to irradiation may reflect certain intrinsic characteristics which allow them to be more or less sensitive. For example, radiation lethality in cells has been correlated with nucleic acid content, structure, and ploidy (11).

Variation in radiation sensitivity of cells may also reflect alterations in the internal mileau. The protective effect of low oxygen tension is an example of this kind of control over radiation response. In mammals, hypoxic hypoxia is known to confer protection by causing significant reductions in oxygen tension of both the spleen and gut. Removing the spleen from pocket mice prior to radiation did not significantly alter their ability to survive a mid-lethal dose. Furthermore, high pressure oxygen treatment, which is known to saturate the tissues with oxygen, overriding pockets of low 0_2 tension, did not enhance the lethality of the delivered dose in these studies. These results suggest that pocket mouse radiation resistance is not the result of fortuitous generalized or local hypoxia occurring during the exposure period.

Hypoxic hypoxia, as administered in this experiment, enhances survival in P. longimembris exposed to a lethal dose of whole body irradiation. In this experiment all of the animals that were administered 2100 rads without hypoxia protection are dead, while all of those surviving exposure to 2100 rads with concomitant hypoxia are alive.

On the basis of the results of these three experiments, we must reject the hypothesis that the pocket mouse survives high levels of acute irradiation by invoking local hypoxia in critical radio-sensitive tissue during irradiation. If hypoxia (i.e., via biochemical or pharmacological mechanisms) was already operating, the mechanism would be "saturated"; therefore, added hypoxia (i.e., via hypoxic hypoxia) would not increase protection.

The suggestion that hypothermia may protract the acute period following irradiation, thereby raising the $LD_{50/30}$ is a valid one. Ultimate recovery is more meaningful than LD_{50} values in instances where hypothermia is possible. However, the fact that several pocket mice did survive 1400 rads, even when forced to maintain a normal active metabolism for the 30-day period, argues against post-irradiation hypothermia playing a large role in Perognathus radiation resistance.

Additional argument against hypothermia causing an artificially high $LD_{50/30}$ in Perognathus is provided by long term survival data. As seen in other mammals, pocket mice that survive the acute phase of radiation sickness normally live months to years following recovery. The slope of as yet incomplete survival curves suggests that life shortening will occur. Long term survivors become totally grey. However, cataracts and tumors are not evident in survivors up to two years after irradiation with dosages ranging up to 1600 rads.

In hibernation, or in artificially produced deep hypothermia, (both produce a hypometabolic state), radiation injury is inhibited until the animal is warmed to normal temperature. The "protective" effect of hypometabolism is only temporary, however, since rewarming radiated animals allows manifestation of the injury in much the same manner as it appears in normometabolic counterparts (23, 24).

There is good evidence that prolongation of survival by hibernation has little to do with recovery from radiation injury. Indeed, it has been demonstrated that animals kept in hibernation after high dose irradiation eventually succumb to radiation injury (23). It is apparent that lowered metabolic rate postpones but does not significantly affect the final outcome of lethal radiation exposure.

In most instances where non-hibernators have been made hypometabolic, the concomitant severe hypoxia has resulted in significant protection (5, 10, 18). This is indicated by the fact that when hypoxia is avoided in these kinds of experiments, protection is not conferred (5). Hypothermia then, may be protective, but only if it is attended by tissue hypoxia.

Moderate alterations in metabolic rate by diurnal or forced activity, cooling, or pharmacologicals, or as a result of metabolic disorders have been shown to influence radiation sensitivity (4, 18, 19, 21). The order of magnitude in changes of radiosensitivity resulting from moderate alterations in metabolic rate suggests that differences in basal metabolic rates among species cannot account for large differences in radiosensitivity observed among mammals (4).

There was no reason to expect (in Perognathus)) that a hypometabolic state during exposure would alter its response to lethal irradiation. Since its radiation response under normothermic conditions is somewhat peculiar, however, it appeared judicious to study the effect of combined hypometabolism and radiation specifically in this species.

As was anticipated, there was no significant difference in survival between normometabolic and hypometabolic Perognathus. The more uniform mortality pattern in the hypometabolic group might have been predicted on the basis of the more uniform physiological state of hypometabolic animals.

Data presented here suggests that pocket mice are just as susceptible to extremely high dose irradiation as conventional mice (13). There is a suggestion that pocket mice may be even slightly more susceptible at the extreme dose levels. Conventional mice and rats survive 24 to 48 hours following doses as high as 20,000 to 30,000 rads; whereas pocket mice survive less than one day.

The plateau showing survival in the dose range from 2000 to 14,000 rads is of great interest. In this dose range pocket mice have a mean survival time of 7 - 8 days, whereas in conventional mice the mean survival time is between 3 and 4 days. In this respect pocket mice are similar to germ-free mice which have a mean survival time of 7.2 days (15).

In general, the response of pocket mice to irradiation appears to be modified in the dose ranges where cell renewal systems are important for survival. Matsuzawa suggests that survival in the "gut death" dose range may be enhanced in germ-free mice by virtue of a prolonged life span of intestinal cells in these animals (15).

Damage to the gastrointestinal mucosa is responsible in a large measure for early deaths in mammals following radiation doses ranging between 1,000 and 10,000 rads. Since the integrity of the gastrointestinal epithelium is contingent upon replacement of cells which are continually being sloughed off, radiation injury incurred at the site of cell renewal is manifested ultimately as villus denudation. The cell renewal system involved, of course, are the cells at the base of the intestinal crypts which are among the most rapidly dividing cells in the body.

Loss of large amounts of body fluid and its consequences such as dehydration and electrolyte imbalance; bleeding into the intestinal lumen; and massive invasion of gut bacteria are among the sequelae of villus denudation. According to Patt and Quastler, large, pleomorphic, functionally abnormal ω_- cells form a coherent sheet lining the intestinal lumen after the initial loss of normal epithelial cells (16). The loss of ω- cells within the normal villus transit time leaves the gut totally denuded leading to "intestinal radiation death I" in 2nd or 3rd day post-irradiation. In some animals ω- cells may persist for several days and death may be due to bacteremia - "intestinal death II" -

between 5 - 7 days in rodents, or replacement of ω - cells with newly formed crypt cells may occur with ultimate complete repair.

Judging from gross pathology, severe gastro-intestinal damage is present in pocket mice receiving doses greater than 1000 rads. It is possible that ω-cells do persist for several days in this animal providing protection during the first days following irradiation. Survival in the 5 - 7 day period may reflect a general resistance to bacteremia common in native rodents. This conforms with the hypothesis of Roderick that general fitness appears to be an indicator of radioresistance in mice (20). Death occurring in pocket mice after the 8th day may be attributed to hematopoietic failure, but is probably enhanced by profound damage in the gut epithelium and capillary net, allowing massive hemorrhaging into the intestinal lumen. This hypothesis is supported by two facts; extremely low platelet counts at the 10th day post-irradiation (6), and autopsy findings.

If this hypothesis withstands further testing, it may be possible to conclude that at least three unrelated mechanisms are acting to protect pocket mice from high dose irradiation. One, persistant ω-cells, aiding the animal in the 1-3 day period; two, natural resistance to common pathogens, enhancing survival in the 5 - 7 day period; and finally, a resistance in bone marrow (possibly rapid regeneration capability) allowing survival in dose ranges that normally are lethal due to hematopoietic death (22).

SUMMARY

A series of P. longimembris were subjected to 1400 rads total body Co^{60} irradiation. One group was administered 100% oxygen at 3 atmospheres during the irradiation. Another was splenectomized prior to irradiation. A third group was forced to maintain its body temperature during the entire 30 day post-irradiation period. Survivors occurred in all groups, suggesting, that taken singly, neither the hypoxia mechanisms or the lowered metabolic rate are responsible for the remarkable radiation resistance of Perognathus.

Perognathus longimembris administered a lethal dose of Co^{60} irradiation while hypometabolic (body temperature $9^{o}C$) responded similarly to normometabolic animals given the same dose. Mean survival time was 10.1 days in the hypometabolic group and 10.8 days in the normometabolic group. Both groups received a single 1700 rads whole body exposure. There was some tendency for deaths to be more closely grouped around the mean in the hypometabolic mice.

Pocket mice were administered 2100 rads total body Co^{60} irradiation while in severe hypoxia. No deaths occurred in the hypoxia protected group. Protected pocket mice administered 2100 rads died in the 8th - 15th day. Hypoxia controls show no death in the same period. It is suggested that the natural radioresistance in pocket mice cannot be explained on the basis of local or general hypoxia invoked during irradiation under standard conditions.

Pocket mice exposed to 8.5 and 13.7 krads massive Co^{60} irradiation had a mean survival time of 7 to 8 days. Those exposed to 24.1 krad had an ST_{50}% of less than one day.

Animals receiving 13.7 and 24.1 krads exhibited ataxia and other behavioral effects immediately following exposure. Autopsies of the

24.1 krad group revealed no grossly observable pathology. Animals in the 13.7 krad group slowly recover from signs of CNS damage, but like those receiving 8.5 krad, succumb within 5 to 11 days. Animals in these two groups showed typical gastrointestinal injury, including massive gastrointestinal hemorrhage.

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SURVIVAL OF POCKET MICE ADMINISTERED Co^{60} RADIATION DURING PERIODS OF HIGH AND LOW METABOLISM

R. G. Lindberg, J. J. Gambino and P. Hayden

INTRODUCTION

Circadian rhythmicity in sensitivity to whole body radiation was demonstrated in Swiss-Webster and C_3H strain mice and in laboratory rats by Pizzarello et al (22,23). Other workers suggest there are no cyclic variations in response to radiation in either CF_1 or ICR mice, or in Sprague-Dawley rats (25,28). It is obvious that in experiments involving biological rhythms, subtle biological and experimentor produced variables might result in conflicting data or interpretation.

Pizzarello reasons that any work designed to confirm or reject the hypothesis that cyclic variation in radiosensitivity occur should be conducted on animals in which the phase and wave-form of the rhythm in question is known with respect to some known point in time, i.e., beginning of photoperiod. He also suggests that concurrent measurements of radiosensitivity and a "marker" rhythm (i.e., locomotion or temperature) are necessary adjuncts to intelligent interpretation (23).

The little pocket mouse, Perognathus longimembris, exhibits well defined daily periodicity in body temperature, metabolic and locomotor activity (12). These are, of course, interrelated parameters. These periodicities have been measured and are documented (13).

In addition to measurements of biological rhythms, we have obtained and reported on measurement of the $LD_{50/30}$ of P. longimembris in response to X and gamma radiation (5).

With this information at our disposal, and with the availability of a large number of P. longimembris maintained under constant environmental conditions, including known photoperiod, we undertook a day vs. night radiation experiment. This species appeared well suited to answer some of the questions posed by previous work of this kind. The objective of this work was to detect differences in survival after irradiation of the mice during their known metabolic low and metabolic high periods.

MATERIALS AND METHODS

Animals used in this experiment were brought in from the field in the Spring. Since these animals were live-trapped, there is no way of knowing their precise age. It is assumed from available field data that the majority of animals (perhaps 80%) trapped in the Spring are juveniles; therefore, the age at the time of irradiation for 80% of the animals used in this experiment was approximately 9 months. The others are assumed to be 1 year 9 months old. Population dynamics studies indicate that in the native state the ecological life expectancy of pocket mice is less than 2 years. Life expectancy in the laboratory is well over 4 years and perhaps as long as 7 years (19).

Pocket mice are housed in air conditioned quarters. Temperature is maintained at 22° $\pm$ 2°C, and relative humidity is 50 $\pm$ 5%. Lights are turned on at 0630 hours and turned off at 0530 hours PST. Mice are housed individually in our laboratory. Other details of animal maintenance were previously reported (13). Maintenance of pocket mice is so simple that they can be left undisturbed for weeks at a time, needing only a handful of dry seeds at 3-4 week intervals.

The metabolic low of pocket mice in our laboratory occurs at Arbitrary Zeitgeber Time* (AZT):02.50. The metabolic high is at AZT:17.00. These times were obtained from observations on a large number of individuals over the past 2 years. As in most other organisms, pocket mice exhibit a great deal of individual variability with respect to activity. Figure 1 shows this variability in a sample group of animals. In order to obtain an experimental group of pocket mice exhibiting a regular rhythm with all of the

* AZT scale runs from 00.00 to 24.00, with 00.00 defined as onset of light.

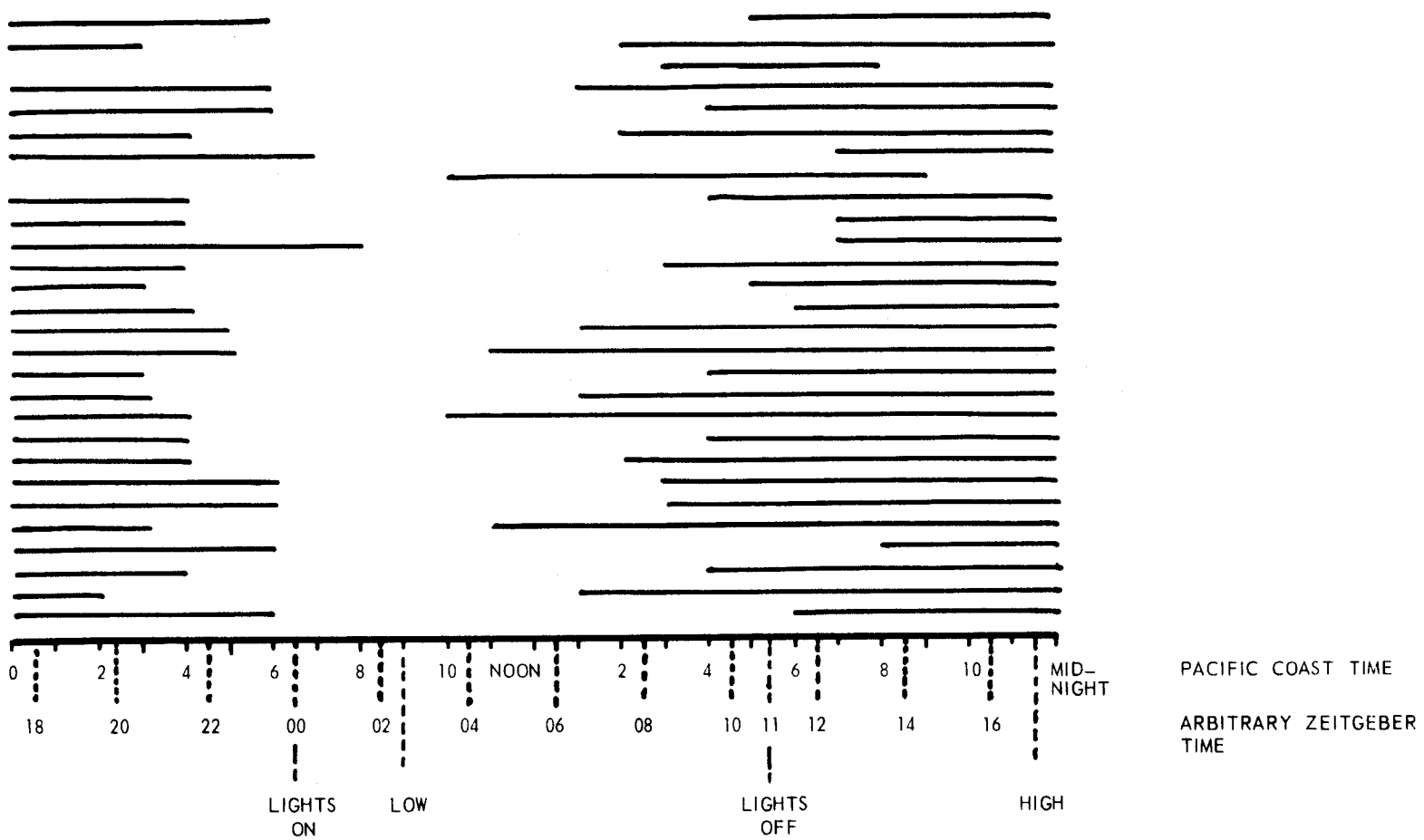

FIGURE 1 PERIODIC LOCOMOTOR ACTIVITY OF POCKET MICE DURING FIRST SEVERAL DAYS IN CONSTANT DARK AFTER PREVIOUS ENTRAINMENT. EACH LINE REPRESENTS ACTIVE PERIOD OF SINGLE ANIMAL

animals in the group in phase with each other, records were kept on over 300 animals for 1 week prior to the proposed radiation. A group of 150 were selected for the experiment on the basis of consistant inactivity during the mid-morning hours. From this group of 150 "in-phase" mice, 5 subgroups of 30 each were established by random methods. Sexes were equally represented in the subgroups.

The experimental design called for irradiation of one group during the metabolic low period, 0900 hours (02.50 AZT), and one group at 2330 hours (17.00 AZT) during their metabolic high. A whole body dose of 1500 rads administered to these two groups is approximately the $LD_{50/30}$ for the species. For purposes of control, one group was administered 1000 rads (approximately $LD_{10/30}$) at 2330 hours. Two sham-irradiated groups were included; one for each exposure period.

One hour before radiation exposure, unanesthetized mice were placed in compartmented plastic boxes and transported to the Co^{60} facility. After irradiation, the animals were returned immediately to the animal quarters and replaced in their original caging.

Changes in the metabolic state of the pocket mice during packaging, transporting and irradiation were minimized by gentle handling. Nevertheless, appearance of the animals during these treatments indicates that their metabolic state was indeed altered. This is especially true of the mice irradiated during their metabolic low period. However, since in this experiment, metabolic state per se was not as important as using it as an indicator of the phasing of other biological rhythms, we were not overly concerned with this point.

Irradiation was administered from a 5000 curie Co^{60} source. Dose rates were 37.8 rads/minute for the 1500 rad dose, and 24.5 rads/minute for the 1000 rad dose. The radiation facility was calibrated with ferric sulphate dosimeters prior to the animal radiations and silver-activated phosphate glass dosimeters were used during the animal exposures. Accuracy of

the dosimetric methods used was $\pm$ 5%. Identical radiation doses to the two 1500 rad groups was assured by precise location of the exposure cages in the Co^{60} room and by accurate timing of the exposure period.

Five mice from each of the five subgroups were sacrificed during the first week post irradiation. Portions of the jejunum and ileum were fixed in formalin, sectioned, and stained with haematoxylin-eosin. The remaining 25 in each group slated for survival studies were left undisturbed but observed several times daily. Time of death was recorded to the nearest 1/4 day. Autopsies were performed as soon as possible after death.

RESULTS

Pocket mice administered 1500 rads whole body irradiation at 0900 hours had an ST_{50} of 11 days. There were no survivors in this group beyond 18 days. Pocket mice administered the same dose under identical conditions except for time of exposure (2330 hours) had an ST_{50} of 15 days. At 30-day post-irradiation, 3 of the night-irradiated group were still alive. Subsequently, one more died at 47 days post-irradiation and 2 remain alive at the end of two months.

Only three mice of the group administered 1000 rads died during the 30-day period. One more died on the 47th day post-irradiation.

No unirradiated controls have died.

Mortality data is presented in Figure 2 as number of animals surviving against time to show the shape of the survival curves.

For analysis, probit transformation of the data were made by plotting per cent survival on probability paper (K & E Probability Scale 359-23) against logarithm of time (Figure 3) (4). A straight line was eye-fitted to the points, giving the greatest weight to those points falling between 10 and 90%. The approximate method of Litchfield was applied to test the difference between mean survival times and slope of the mortality curves of the two 1500 rad irradiated groups (14,15)*.

Because there are many inherent errors in experiments of this sort, the $p < 0.02$ probability level was chosen as a test of significance (10).

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* See appendix for complete analysis

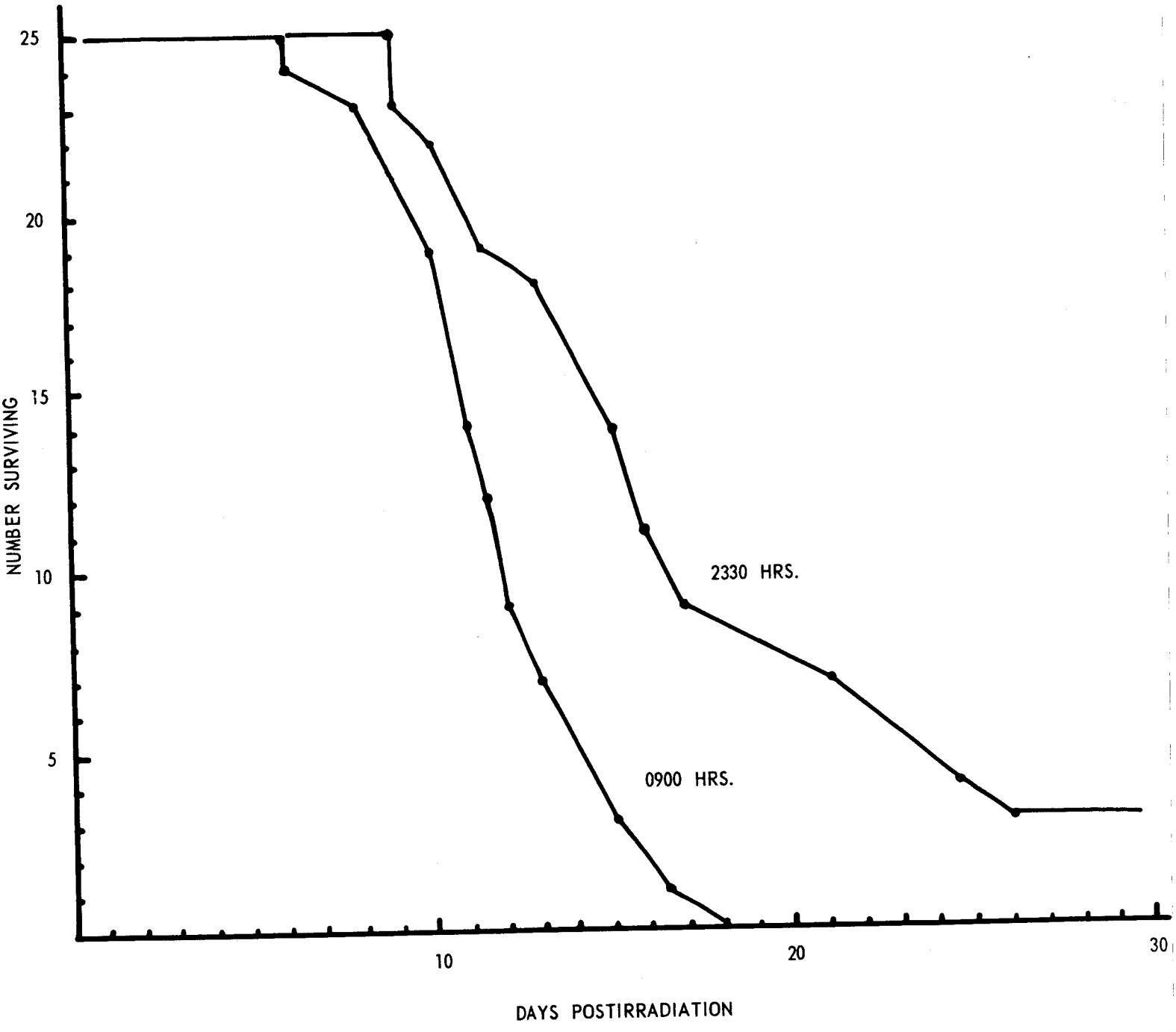

FIGURE 2 SURVIVAL IN TWO GROUPS OF POCKET MICE ADMINISTERED WHOLE BODY Co^{60} RADIATION AT TWO DIFFERENT TIMES OF DAY

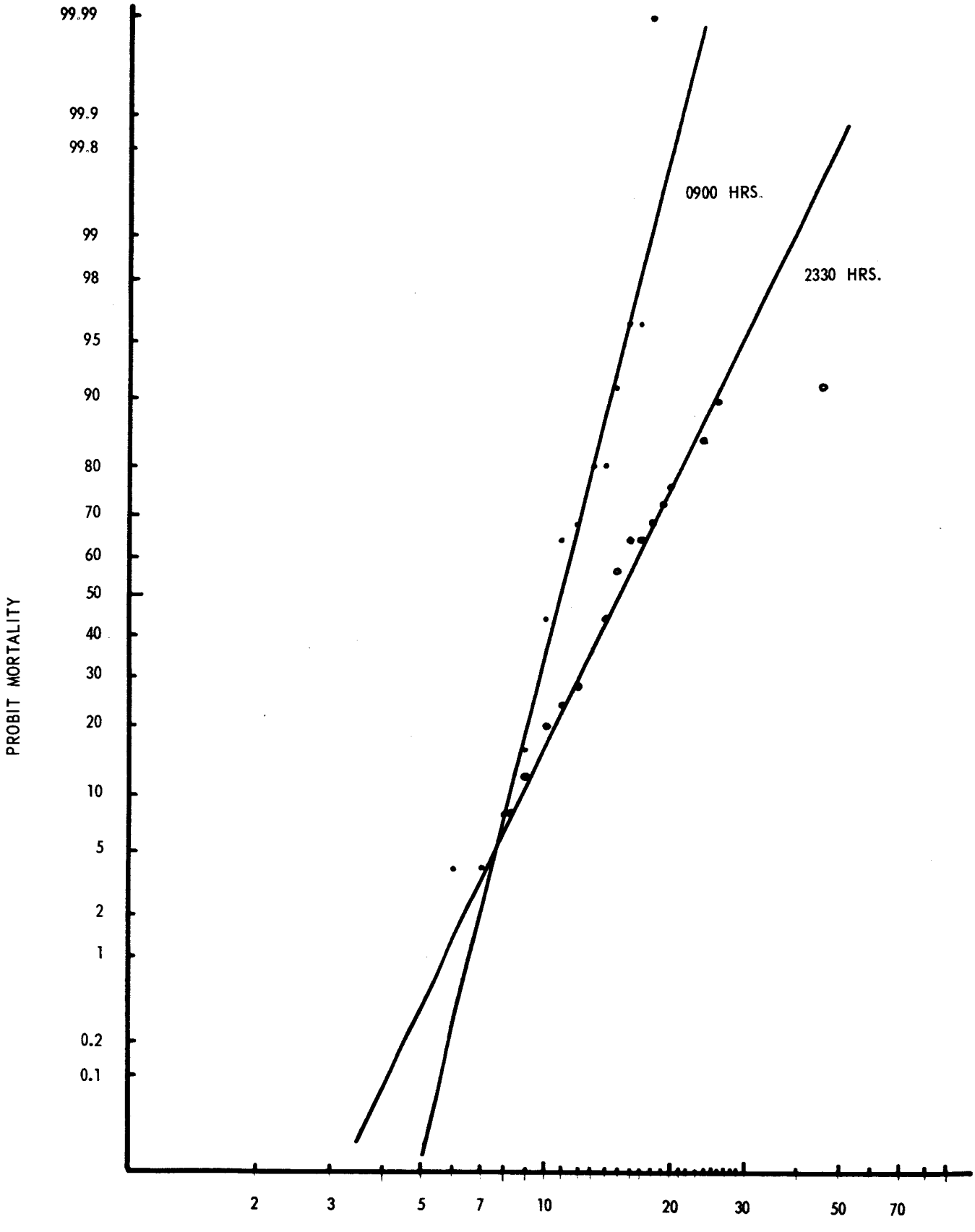

FIGURE 3 PROBIT TRANSFORMATION OF SURVIVAL DATA OF POCKET MICE ADMINISTERED 1500 RADS CO^{60} RADIATION AT TWO TIMES OF DAY

At the $p < 0.02$ probability level the two mean survival times are significantly different and the two curves deviate significantly from parallelism.

It can be stated with reasonable confidence that day-night variation in response to whole body irradiation occurs in the little pocket mouse, P. longimembris. Pocket mice irradiated during the period of metabolic high live significantly longer than those irradiated during the metabolic low.

The pattern of gross autopsy findings were similar in the two groups studied. As reported earlier, hematopoietic failure, particularly failure of platelet production appears to be the chief cause of death (5). Most deaths in pocket mice at this dose level occur between the 7th and 16th days with severe gastrointestinal bleeding found in more than half of the dead animals. Approximately half of the animals exhibited some form of moderate or severe cerebral vascular hemorrhaging, irrespective of whether gastrointestinal hemorrhage was present. Respiratory involvement as judged by hemorrhagia, consolidation, or complete hepatization was seen in about 20% of the dead post-irradiation animals. Bleeding in areas other than the cranium or gut occurred in less than 5% of the radiation-exposed animals. Gastrointestinal damage as judged by liquification of the intestinal tract and rapid autolysis after death without concomitant hemorrhage appeared in several animals.

Study of histologic preparation of jejumen and ileum taken from sacrificed animals is in progress and results will be reported at a later time.

DISCUSSION

Under the conditions of this experiment mortality in day versus night irradiated pocket mice differed significantly ($p < 0.02$). Survival of the night irradiated group was significantly enhanced as judged by both mean survival time and the slope of the mortality curve of the two groups.

This enhanced survival of night-exposed animals extends beyond the 30-day acute post-irradiation period, with 2 of 25 initial animals still alive two months after exposure.

With respect to the presence of a difference in response to irradiation delivered at different periods of the activity cycle, these results are in concordance with those of Pizzarello et al (23). According to these authors Swiss-Webster and C_3H strains of mice are more sensitive to whole body x-irradiation given at 2 a.m. (AZT 19.00) than at any other time in the diurnal cycle. Earlier they had reported similar results in rats, with greater sensitivity when irradiated at 9 p.m. in contrast to 9 a.m. (22).

Although we demonstrate an opposite effect (i.e., greater sensitivity of pocket mice irradiated at 9 a.m. AZT 02.50), our results support the generalization that a time of exposure related difference in response to whole body radiation exists in certain rodents. This is contradictory to the views of Rugh (26) and Straube (29) who argue from their data that no significant difference in survival of CF_1 mice and Sprague-Dawley rats occurs when these animals are irradiated at different times of the day. Pizzarello et al conclude that statistical treatment of Rugh's data was inadequate and a significant difference was demonstrated, but went undetected by the authors (23).

Although Rugh did not confirm the existence of diurnal variation in sensitivity in CF_1 mice, he did concede that the idea of minor diurnal fluctuations in radiosensitivity is plausible. This, he notes, is suggested by a slightly higher mortality in his mice irradiated at 6 a.m. than at noon. He explains this difference as being the result of night activity which normally occurs in these animals. To substantiate this argument Rugh uses the work of Kimeldorf et al in which exercise to exhaustion was shown to enhance the deleterious effect of irradiation (8,9). Benjamin and Peyser suggest similar response in mice administered lethal irradiation during exploratory activity or forced activity (1).

Vacek, on the other hand, proposes an opposite effect of normal physiological activity on radiation response (30). According to Vacek there is a positive correlation between oxygen consumption during irradiation and survival time. He shows that rats having a lower oxygen consumption during irradiation die sooner than those having a higher oxygen consumption.

In this respect we are in closer accord with Vacek than with Rugh or Benjamin. Pocket mice having a higher mortality when irradiated during the morning hours are assumed to have a lower average oxygen consumption than their night counterparts. They also had a shorter survival time. Since we did not measure oxygen consumption during irradiation, we can only suggest a relationship between metabolic activity and survival in pocket mice. Michaelson and Odland propose that among the mammals, metabolic rate and recovery from radiation injury might be related (18). Other work, however, indicates that minor fluctuations in metabolic rate as judged by body temperature, unless accompanied by hypoxia, may have very little bearing on radiation response (3,6,7,24). In attempting to elucidate the cyclic nature of radiation response, metabolic rate may be useful as an index of other cyclic activity within the organism. Pizzarello suggests that metabolic or locomotor activity may be useful as a "marker" when studying cyclic radiosensitivity (23). But, according to him, the ultimate search should be for physiological rhythms which may be directly related to radiosensitivity at a given phase in a given photoperiod.

It appears that this search should be directed toward elucidating circadian rhythms in cell activity. A massive literature exists on mitotic indices in various body tissues (17). Cell population kinetics of bone marrow and intestinal epithelium, important regenerative tissues, have been especially well studied (21). In vitro studies on synchronously dividing cell cultures reveal differences in radiosensitivity relative to the phase of the cell cycle exposed to irradiation (11). More recently, this has been corroborated in vivo (20).

The level of mitotic activity of similar tissue may be strikingly different between species and strains (2). Some reports suggest that mitotic activity in various mammalian tissues occurs in waves which may be circadian (17,27,28). These factors, operating singly or together, could result in the tremendous range of LD_{50} values reported for various mammals. Matsuzawa, for example, suggests that germfree mice have an ST_{50} of 7 days compared to 3 days in conventional mice following a lethal dose of irradiation, because germfree mice have a slow turnover rate of intestinal epithelium (16). In terms of cell population kinetics a low mitotic index might provide a situation in which fewer cells are in the sensitive phase of their cycle or it may provide greater opportunity for repair. If this were the case, it would not only lengthen survival time but it would also increase absolute survival in a group of animals. The natural radiation resistance of pocket mice and, indeed, other rodents may be explicable on this basis (5).

In a like manner, diurnal or seasonal cyclic rhythms in mitotic activity of gut epithelium or hematopoietic tissue may cause daily or seasonal differences in radiation response within a given species or strain. Preliminary estimates of pocket mouse intestinal epithelial mitosis reveal more post-metaphasal mitotic figures in night-sacrificed than in day-sacrificed animals.* If this difference is corroborated by further work, it will suggest the possibility that night irradiation catches the majority of pocket mouse intestinal crypt cells in the G_1 phase, when, according to

* Work currently underway in this laboratory.

most, they are least sensitive to irradiation (20,11). Certainly, longer survival of night-irradiated mice in this study favors this point.

In any case, the fact that initial studies in pocket mice show diurnal differences in mitotic activity of a critical radiosensitive tissue, provides enough incentive to extend work on the relationship of mitotic activity in vital tissues and whole body radiation sensitivity.

Comparison of mortality between current and previous pocket mouse irradiations in this laboratory reveals a higher mortality in the current group. The 1500 rad dose used in this experiment is considered the $LD_{50/30}$ for this species (5). However, a 1500 rad dose produced 100% mortality in 18 days in the day-irradiated group and 94% mortality in 30 days in the night-irradiated group. In contrast, the group receiving 1000 rads in this experiment had approximately the same mortality as was reported earlier for pocket mice receiving that dose.

The differences observed may be the result of normal seasonal variability in response to irradiation (25). Pocket mice have been observed to undergo seasonal changes in frequency of spontaneous torpor, suggesting underlying changes in their physiological state (13).* These changes could cause seasonal variation in response to irradiation.

* Also see Part II of this report.

SUMMARY

Pocket mice, Perognathus longimembris, administered lethal whole body gamma irradiation at two different times of a day show different response to irradiation. Those irradiated in the morning during their period of low metabolism had an ST_{50} of 11 days. Those irradiated at night during their metabolic high period had an ST_{50} of 15 days. The morning irradiated animals were all dead by the 18th day post-irradiation. Several night-irradiated animals were alive at 30 days post-irradiation. The relationships of radiation sensitivity to metabolic activity, mitotic activity and cell population kinetics of critical regenerative tissues is discussed.

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APPENDIX I

Statistical Analysis

TIME - PER CENT EFFECT CURVES

A sigmoid curve becomes straight when plotted as cumulative per cent effect in probits as the ordinate, against time in logarithm units, is the abscissa. It has been shown that the provisional graphic solution of data plotted in this manner can be made so accurately that it generally suffices (14,15).

In the Litchfield method a straight line is visually fitted to the data points plotted on probability-logarithmic paper. The greatest weight is given to points above 10% and below 90%.

With the data plotted in this manner the logarithm of the Median Effective Time (log ET_{50}) and Standard Deviation can be estimated directly. Then by simple computation or by use of a system of monographs, tests for significance of two reaction times or parallelisms between curves can be performed.

LITCHFIELD METHOD

Logarithmic Parameter and their Standard Errors for 98/100 Probability

(1) Logarithm of Median Effective Time (ET_{50}), (Median Survival Time) (ST_{50})

Log ET_{50} = log time at which line intercepts 5.0 probits

(2) Standard Deviation of the Log Times

$$\sigma = \frac{X_2 - X_1}{Y_2 - Y_1}$$ where X_1, Y_1 and X_2 Y_2 are widely separated points on the line relating probits (Y) and log doses (X).

(3) Standard Error of log ET_{50} (98/100 probability)

$$SE_{\log ET_{50}} = \frac{2.33 * \sigma}{\sqrt{N}}$$ where N = No. individuals tested

(4) Standard Error of the Standard Deviation, σ

$$SE = \frac{2.33\sigma}{\sqrt{2N - 1}}$$

Arithmetic Parameters and their Confidence Limits for 98/100 Probability

(1) Median Effective Time (ET_{50}) (ST_{50})

ET_{50} = time at which line intercepts 50%

(2) Slope Function (Equivalent of the Standard Deviation)

$$S = \frac{ET_{84}/ET_{50} + ET_{50}/ET_{16}}{2}$$

where S = antilog s

and ET_{84} is time at which line intercepts 84% etc.

* NOTE: Confidence coefficient (Z_c) may be changed to conform with level of probability required -

confidence level	99.73%	99%	98%	95%
Z_c	3.00	2.58	2.33	1.96

(3) The Factor for the ET_{50}

Confidence limits obtained by multiplying and dividing the estimate of the parameter by a factor (f).

Or in logarithms - add Standard Error to or subtract Standard Error from the logarithm of the parameter.

In other words, f is equal to the antilog of the Standard Error

$$f_{ET_{50}} = \text{antilog } \left(\frac{2.33\sigma}{\sqrt{N}}\right)$$

$$= S^{\frac{2.33}{\sqrt{N}}}$$

(4) The Factor for S

$$f_S = \text{Antilog } \left(\frac{2.33}{\sqrt{2N - 1}}\right)$$

$$= S^{\frac{2.33}{\sqrt{N - 1}}}$$

Truncated Data

N = No. individuals tested

$N_1 = 2N - 1$

N_2 = N Corrected for Truncated data

$N_3 = N_1$ Corrected for Truncated data

Use nomograph for obtaining N_2 and N_3

Substitute N_2 for N

N_3 for N_1

Otherwise method is identical

Reaction Time Ratio RR Test for significance of ET_{50}

$$RR = \frac{ET_{50_1}}{ET_{50_2}} \quad \text{where } ET_{50_1} \text{ is larger value}$$

$$f_{RR} = \frac{fET_{50_1}}{fET_{S_2}}$$

RR should exceed f_{RR} if the two median raction times are significantly different

Slope Function Ratio; SR - Test for parallelism

$$SR = \frac{S_1}{S_2} \quad \text{where } S_1 \text{ is larger value}$$

$$f_{SR} = \frac{f_{S_1}}{f_{S_2}}$$

$SR > f_{SR}$ curve deviates significantly from parallelism

CALCULATIONS OF σ AND S BY LOGARITHMS

GROUP A - Night Irradiated	GROUP D - Day Irradiated
90% X_2 = 1.40154	1.16137
Log days 10% X_1 = 0.93952	0.91645
$X_2 - X_1$ = 0.46702	0.24492
90% Y_2 = 6.28	6.28
Probit 10% Y_1 = 3.72	3.72
$Y_2 - Y_1$ = 2.56	2.56
$\sigma = \frac{X_2 - X_1}{Y_2 - Y_1} = \frac{0.46702}{2.56}$	$\frac{0.24492}{2.56}$
σ = 0.182	σ = 0.0956
Antilog σ = 1.52	Antilog σ = 1.25
S = 1.52	S = 1.25

CALCULATION OF S BY SLOPE FUNCTION

$S = \frac{ET_{84/ET_{50}} + ET_{50/ET_{16}}}{2}$	$S = \frac{ET_{84/ET_{50}} + ET_{50/ET_{16}}}{2}$
$= \frac{22.5/15.0 + 15/9.75}{2}$	$= \frac{13.5/11.0 + 11.0/8.75}{2}$
$= \frac{1.50 + 1.53}{2}$	$= \frac{1.23 + 1.26}{2}$
S = 1.52	S = 1.25

GROUP A	GROUP D

$$SE_{\log ET_{50}} = \frac{2.33\ \sigma}{\sqrt{N}}$$

$SE_{\log ET_{50}} = \frac{2.33\ \sigma}{\sqrt{N}}$	
$= \frac{(2.33)\ (0.182)}{5}$	$= \frac{(2.33)\ (0.096)}{5}$
$= \frac{0.424}{5}$	$= \frac{0.224}{5}$
$= 0.0848$	$= 0.045$

Factor for ET_{50} = Antilog $SE_{\log ET_{50}}$

$f_{ET_{50}} = 1.213$	1.109

Factor for $ET_{50} = S^{\frac{2.33}{\sqrt{N}}}$

$f_{ET_{50}} = 1.52^{0.466}$	$= 1.25^{0.466}$
$\log f_{ET_{50}} = 0.466 \log 1.52$	$= 0.466 \log 1.25$
$= (0.466)\ (0.18184)$	$= (0.466)\ (.09691)$
$= .08473$	$= .04516$
$f_{ET_{50}} = 1.213$	$= 1.109$

GROUP A		GROUP D
$SE_\sigma = \frac{2.33\ \sigma}{\sqrt{2N - 1}}$		
$SE_\sigma = \frac{(2.33)\ (0.182)}{\sqrt{49}}$		$= \frac{(2.33)\ (0.0950)}{\sqrt{49}}$
$= 0.061$		$= 0.032$

Factor for S		$S \frac{2.33}{\sqrt{2N - 1}}$
f_S = antilog (SE σ)	or	
$= 1.151$		$= 1.076$
or		
$= (.333)\ (0.18\ 184)$		$= (.333)\ (0.09\ 691)$
$\log f_S = .0605$		$= 0.032$
$= 1.15$		$= 1.076$

CORRECTION FOR TRUNCATED DATA

GROUP A	GROUP D
N	25
N_1	49
N_2* 24.5	
N_3* 42.0	
$SE_\sigma = \frac{(2.33)\ (0.182)}{\sqrt{42}}$	
$= \frac{0.42406}{6.48}$	$f_{SR} = \frac{f_{S_1}}{f_{S_2}} = \frac{1.163}{1.076}$
$= 0.0654$	
$f_S = 1.163$	$= 1.081$
$SE_{\log ET_{50}} = \frac{(2.33)\ (0.182)}{24.5}$	
$= \frac{0.42406}{4.95}$	$f_{RR} = \frac{f\ ET_{50}}{f\ ET_{52}} = \frac{1.218}{1.109}$
$= 0.0856$	$= 1.098$
$f_{ET_{50}} = 1.218$	

* From nomograph #4 in Litchfield and Wilcoxon (1949)

SUMMARY OF STATISTICAL DATA COMPUTATION

Parameter	Group A	Group D	Ratios	Test of Significance
ET_{50}	15	11		
log ET_{50}	1.17609	1.04139		
$f_{ET_{50}}$	1.213 1.218*	1.109		
RR			1.364	
f_{RR}			1.094 1.098*	RR > f_{RR}
S	1.52	1.25		
f_S	1.151 1.163*	1.076		
SR			1.216	
f_{SR}			1.0697 1.081*	SR < f_{SR}

* Corrected for truncated data.

The Circadian Rhythm of Metabolic Rate in Pocket Mice, Particularly Perognathus longimembris.[1,3]

by

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Abstract

The rate of oxygen consumption of Perognathus longimembris was measured under different conditions of ambient temperature (T_a), lighting, food supply, composition of the atmosphere, and physiological conditioning. In all instances the pocket mice showed a rhythm of metabolism with a periodicity that was not significantly different from 24 hours. The amplitude of the rhythm, its phasing with respect to the light cycle, and the relative duration of the phases of low and high metabolism did vary. In September, P. longimembris kept at T_a 24°C with food had a metabolic rhythm closely synchronized with the light cycle (high at night and low in the day), and a low incidence of hypometabolic periods (on 12.5% of days). In early May, mice at T_a 22°C tended to be out of synchrony with the photoperiod and were hypometabolic during 32.5% of the days. Metabolic lability probably varies seasonally. Periods of torpor may influence the establishment of synchronization of activity with the light cycle in springtime when pocket mice are becoming active on the surface after a period of winter

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2 - Dept. of Biological Sciences, Univ. Southern California, Los Angeles

3 - This manuscript has been submitted for publication to the Journal of Mammalogy

seclusion. The metabolic rhythm at T_a 35°C was the same as at 24° except that the phase of high metabolism tended to continue into the light period. Probably this is a metabolic overshoot due to hyperthermia of the mice when they are active at this high T_a. When mice were deprived of food at T_a 22°C they had a 100% incidence of daily torpor and a deeper hypometabolism than mice that were not starved. Their first periods of torpor were advanced into the first dark period, and the metabolic rhythm "reset" itself on this basis. When mice were exposed to the greater stress of starvation at T_a 10°C, all mice lost the pattern of daily arousals to normal metabolism, and remained torpid for one or more days. In some instances the mice remained at a level of deep hypometabolism (< 0.5 ml O_2/g hr) for 43 to 56 hours, and then spontaneously aroused. However, a basic circadian pattern did persist. There was a metabolic rhythm with a very suppressed amplitude, and/or arousals occurred on some whole multiple of 24 hours. Mice starved at 10°C had a lower mean minimum hypometabolic rate than those at 22°C. A circadian metabolic rhythm persisted in mice that were cold-conditioned, in heavyweight animals, in mice kept in continuous darkness with or without isolation from external sounds, and in mice kept in an air atmosphere with 4.5% CO_2. The cold-conditioned mice had the shortest phases of normal metabolism and the longest phases of sustained deep hypometabolism. The heavyweight animals had the longest mean duration of normal metabolic phases. Acute whole-body gamma radiation of 1400 r did not alter the periodicity or phasing of the metabolic rhythm of mice at T_a 22° and 35°C. P. longimembris (mean wt 10.7 g), P. inornatus (14.6g) and P. formosus (20.5 g) were compared at T_a 10°C, without food, and in continuous darkness. The results suggest that at 10°C metabolic lability and tolerance for a hypometabolic state vary inversely with body size for these species. The persistence of a circadian metabolic rhythm, suppressed or not, has adaptive value in hibernating animals if it promotes the arousal of an animal with its activity pattern in proper phase with the light cycle, and/or if it insures sufficient arousals to properly correct the physiological changes that occur during torpor.

Introduction

Many species of mammals have rather strictly defined nocturnal or diurnal activity patterns in nature. This is one of the obvious functional organizations of biotic communities. In a few instances (e.g. Pearson, 1960) activity rhythms have been quantitatively studied with the individual mammals free-living in nature. Many species have been studied in the laboratory and been shown to have activity patterns that persist in captivity. Aschoff (1962) reviews this work.

Circadian rhythms of behavior are usually adaptively phased with periodicities of environmental factors, and are able to shift in time to reestablish normal phase relationships when external factors are experimentally altered. As is to be expected, activity rhythms are accompanied by rhythms of body temperature and metabolic rate, and other physiological processes have the same circadian periodicity. Because of the integration of physiological processes, these different rhythms have definite phase relationships with each other (see Halberg, 1960).

It is not surprising, therefore, to find a metabolic rhythm in any particular taxonomic group of mammals, such as the genus Perognathus. The rhythm of P. longimembris is noteworthy, however, because of the extreme amplitude of the cycle, and because there is little information in the literature on metabolic cycles per se in mammals. Pearson (1947) found night-day ratios of oxygen consumption ranging from 1.17 to 4.0 for seven species of small mammals, and lesser ratios of 1.0 to 1.09 for eight other species. The ratios are in agreement with the activity patterns of the

species. Circadian rhythms of metabolism have been demonstrated for Perognathus californicus (Tucker, 1962), Myotis myotis and Glis glis (Pohl, 1961) and Citellus tereticaudus (Hudson, 1964). Hart (1950, 1952) has studied the diel metabolic cycle of white mice, and Heusner (1957) the cycle of white rats.

A major challenge to students of rhythms is the discernment of the mechanisms of timing and the bases of coordination of biological rhythms. Much work has been directed to this end (for example, Withrow, 1959; Biological Clocks, 1960; Rhythmic Functions in Living Systems, 1961; Aschoff, 1963; Bunning, 1964). The observation that a rhythm may persist in the absence of obvious environmental cues, i.e., light and temperature cycles, suggests that the rhythm is inherent in the organism, and/or is dependent upon less obvious environmental periodicities. The special metabolic nature of Perognathus longimembris, and its small size, present a promising situation for experimental study of rhythm of homeothermic organisms while they are in prolonged orbits in space, beyond the "reach" of terrestrial cues.

Materials and Methods

Specimens. Live specimens of Perognathus were collected at Whitewater Canyon, Deep Springs Valley, Barstow and Pearblossom, California, and from Lathrop Wells, Nevada.

Mice were housed individually in wide-mouth gallon jars, with a substrate of sand or granulated adsorbent clay. A mixture of parakeet seed, rolled oats and sunflower seed was provided in surplus; small amounts of vegetable greens were given occasionally. The animal room was kept at 20 to 24°C, 45 to 55% relative humidity, and photoperiod from 1600 to 1800 hours PST.

Metabolic measurements. Oxygen consumption of the pocket mice was measured in two ways: (1) in an open-system with a Beckman G-2 Paramagnetic Oxygen Analyzer (POA), (2) in a closed-system automatic respirometer (Metabolor).

With the Beckman POA, the oxygen content of an airstream passing from an animal chamber is continuously measured and recorded. Only one animal, or a group of mice collectively, can be measured at a time. Oxygen consumption, as ml $O_{2_{stp}}$/g hr. is calculated from the rate of air flow, change in oxygen content, and animal weight.

The custom built Metabolor has nine independent closed-system units. In each unit an oxygen-containing atmosphere is continuously circulated through the animal chamber, and carbon dioxide and water vapor absorbers. As oxygen is consumed, the pressure drops within the sytem; after a unit

drop in pressure, a sensing manometer triggers a solenoid system, and oxygen is replaced. The time of each refilling is recorded by an event recorder. Oxygen consumption is calculated from the known volume of the unit refill, the number of refills per unit of time, and the weight of the animal. Refill volumes of about 20 ml and 10.5 ml were used, with 1000 cc and 280 cc animal chambers respectively. With both methods, the animal chambers are kept in a constant temperature water bath.

Since the study was oriented towards the use of Perognathus for biosatellite experiments in which it is technically desirable to have a one-gas system, most of the measurements in the Metabolor were made with an atmosphere varying from 90 to 80% oxygen, 10 to 20% nitrogen, at 760 mmHg pressure. Some measurements were made with the mice in a normal air atmosphere. A series of tests strongly suggests that an 80 to 90% oxygen atmosphere has no effect on the metabolic rates of P. longimembris, over periods of at least 7 days exposure. However, there is evidence of an effect on health and survival during longer exposures.

The Metabolor method has the advantage of being able to provide results on nine animals simultaneously and measurements can be made conveniently over as long a time as desired. The Metabolor cannot measure short-term changes in oxygen consumption, being limited to the time needed for an animal to consume 10.5 or 20 ml of oxygen and thus record an event. The Beckman POA method measures oxygen consumption continuously, so that all changes are recorded. The humidity of the airstream flowing through the chamber in the POA method can be regulated to any desired value, while only dry air can be used in the Metabolor. The two methods of measurement are complementary, and they give comparable results.

Experimental conditions. Metabolic rates were measured with the animals kept at ambient temperatures of 10 to 35°C, with a 12-hour photoperiod (0600 to 1800 hours PST) or in continuous darkness, with or without food, in dry, moist or saturated air, in an atmosphere with 21% oxygen or 80 to 90% oxygen, with no carbon dioxide or with 4.5% carbon dioxide in air,

with exposure to laboratory noises or isolated from external sounds. Most measurements were for individual mice, but some records were made from groups of six mice. Two groups of mice were studied after they had been given an acute whole body gamma radiation of 1400 r from a cobalt 60 source.

Criteria for delimiting metabolic periodicities. The periodicity of an event can be measured most accurately when it is quite discrete or qualitatively different from adjacent events, i.e., when at any particular time the event clearly is or is not occurring. For example, the activity of a rodent outside its nest box is such a discontinuous situation. Since metabolism is a continuous phenomenon, the measurement of a metabolic rhythm is going to be inherently imprecise. Rate categories can be set up, e.g., normal metabolism and light, moderate and deep hypometabolism, but they are always arbitrary to some extent. Therefore, it is often impossible to clearly define the beginning and end of the low and high phases of a rhythm. The variability of measurements leads to instances of statistical insignificance in comparisons of results which may be hiding biologically significant differences.

In the present study we have preferred to use the midpoints of periods of high or low metabolis as the landmarks in the calculation of periodicities. Midpoints can be estimated with greater reliability than the limits of a period of change, and midpoints can be expected to be more constant than either the beginnings or ends of metabolic phases. When an animal spends most of its time in a normal metabolic state, then the midpoints of the metabolic lows are the most discrete events in a record. When an animal is hypometabolic for most of the time, then it is the periods of arousal that are the most obvious rhythmic occurrences.

In the analyses of measurements on metabolic rates, the threshold of hypometabolism is taken as the minimum maintenance metabolic rate, determined as: $Y = 10.127 - 0.279X$, where Y is ml O_2/g hr, and X is ambient temperature over the range of 2 to 32°C. This relationship is calculated from the results of Chew et al. (1963).

At T_a 22° and 24°C, the hypometabolic thresholds are 4.0 and 3.4 ml O_2/g hr respectively. Other metabolic categories at these temperatures are: light hypometabolism, threshold to 2.5 ml; moderate hypometabolism 2.5 to 1.0 ml, deep hypometabolism <1.0 ml O_2/g hr. At 10°C light hypometabolism is 7.3 to 2.0 ml, moderate hypometabolism 2.0 to 0.5 ml, and deep hypometabolism <0.5 ml O_2/ g hr.

Results and Discussion
Experiments with Perognathus longimembris

Mice kept under "moderate" conditions. The mice of Groups 1 and 2 (see Table 1) were kept at 24 and 22°C respectively, with food, on a 12-hour photoperiod, and in a dry atmosphere of 80 to 90% oxygen.

In Group 1, measured September 6 to 10, six of seven mice showed a clear metabolic rhythm, in phase with the photoperiod. The mean interval between midpoints of metabolic lows was 23.9 hrs. In all instances, the daily minimum rate occurred during the light period; the mean midpoint of periods of low metabolism was 1300 hours. In half the instances the period of low metabolism was completely within the light, while in half it persisted into the dark phase of the lighting cycle, but not longer than 3 hours.

As shown in Figure 1, there were two types of rhythms, with respect to their amplitudes. In one type the pocket mouse never became hypometabolic. At night, during its activity period, the mouse's metabolism was considerably above the hypometabolic threshold, while during the day, metabolism declined rather abruptly to near this threshold. In the other pattern the animal became hypometabolic during one or more daylight periods. For Group 1 as a whole, metabolic rates dropped into the hypometabolic range during 12.5% of the periods of low metabolism.

One of the seven mice in Group 1 did not show a metabolic rhythm. This animal died soon after the end of the measurement period, and it is not included in Table 1.

Table 1. Summary of measurements for Different Groups of P. longimembris.

Experimental conditions	Period of metabolic rhythm mean ± SE (=range), in hrs	Mean duration in hrs (=n)	Incidence of period of low metabolism, % within light	Light→ dark	within dark	dark→ light	Incidence of hypometabolism % of days	Mean minimum hypometabolic rate ml O_2/g hr
Group 1. Sept. 6-10, 1962. n=6/7[a] 24°C, with food 12-hr photoperiod, dry 80-90% oxygen	23.9 ± 0.28[b] (22.5-25.3)	Lows[e] 9.1	50%	50%	0	0	12.5	
Group 2. April 30 - May 14 1963. n=9/9. 22°C, with food, 12-hr photoperiod, dry 80-90% oxygen	25.8 ± 0.77[c] (15.1-33.7)	Lows 5.3	57.1	7.1	14.3	21.4	32.5	0.52 (0.33-0.82)
Group 3. Nov. 6-12,1963. n=7/7 35°C, with food and water, 12-hr photoperiod, dry air	24.1 ± 1.02[b] (13.7-32.1)		89.9	0	11.2	0	0	
Group 4. May 21-25,1963. n=7/7 22°C, without food, 12-hr photoperiod, dry 80-90% oxygen	24.8 ± 0.67[d] (21.0-28.9)		12	20	12	56	100	0.32 (0.13-0.46)
Group 5. Feb. 19-26.1963. n=6/9 10°C, without food, 12-hr photoperiod, 80-90% oxygen saturated with water vapor	23.2 ± 0.57[d] (17.9-25.3)	MDH[e] 50.0 (3) NM[e] 5.7 (18)	17.6	47.1	35.3	0	100	0.12 (0.06-0.16)
Group 6. July 25-30,1963 n=5/8 cold conditioned animals, 10°C, without food, 12-hr photoperiod, dry air.	23.9 ± 0.65[d] (20.9-26.8)	MDH 56.1 (5) NM 2.35 (9)	11.1	33.3	55.5	0	100	0.11 (0.05-0.20)
Group 7. July 31 - Aug. 7.1963. n=7/8 heavyweight animals, 10°C, without food, continuous darkness, dry air	23.7 ± 0.79[d] (20.3-27.9)	MDH 47.9 (5) NM 12.0 (8)	0	75	25	0	100	0.10 (0.06-0.10)

Table 1 (continued)

Group 8. Nov. 26- Dec. 4, 1962 10°C, without food, continuous darkness,dry air.								
8a, isolated from sound, n=4/4	24.7 ± 0.28[d]						96.8	0.10 (0.08-0.12)
8b, not isolated from sound, n=4/4	25.5 ± 0.79[d]						92.9	0.12 (0.09-0.16)
Group 9. March 11-25,1963, n=6/6 6 mice in series. 10°C, without food, 12-hr photoperiod, nesting material, dry air with 4.5% CO_2	22.25[d]							
Group 10. 22°C, with food, 12-hr photoperiod, dry 80-90% oxygen. 1400 r whole body exposure.								
March 25-April 1 1963 n=7/7	24.6 ± 0.56[b]	14	24	8	54		54.8	0.48 (0.33-0.73)
April 1-8,1963. n=8/8	24.0 ± 0.49[b]						51.8	
Group 11, 35°C, with food and water, 12-hr photoperiod, dry air, 1400 r whole body exposure								
Nov. 20-27, 1963. n=8/8	23.5 ± 0.99[b]	62.8	0	37.2	0		0	
Nov. 20 - Dec. 18, 1963. n=4/8	24.0 ± 0.28							

a - surviving number/initial number; b - period calculated on basis of midpoints of all phases of low metabolism whether within normometabolic or hypometabolic range; c - period calculated on basis of midpoints of phases of hypometabolism; d - period calculated on basis of midpoints of periods of deep hypometabolism; e - MDH, multiple deep hypometabolic periods, sustained for more than one day, NM, normometabolic phases. Lows, periods of low metabolic rate (usually within normal range).

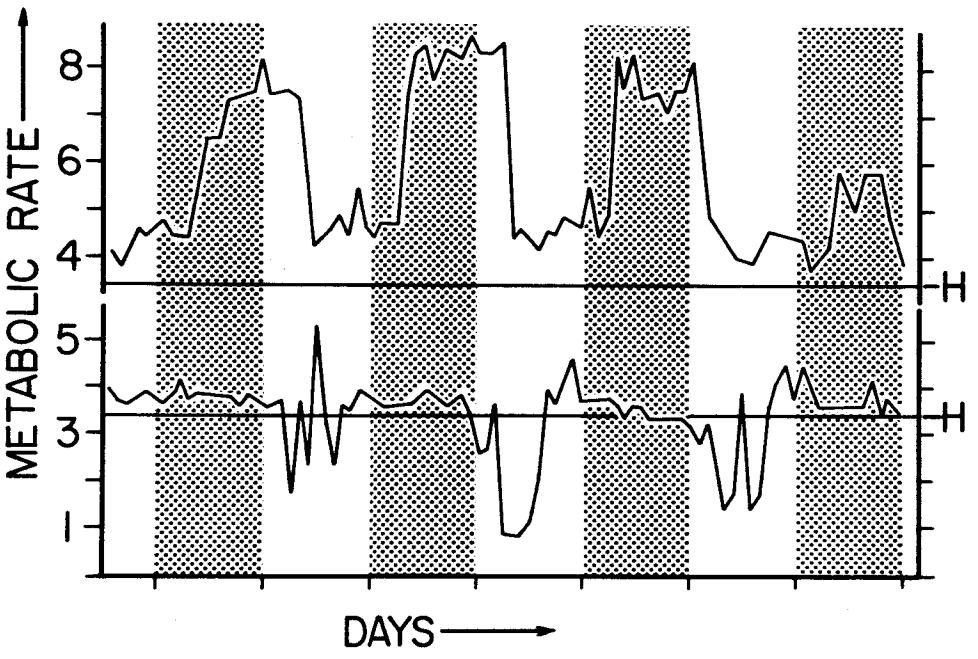

Figure 1. Metabolic rhythms at 24°C. Two representative P. longimembris of Group 1. Metabolic rate as ml O_2/g hr; H is hypometabolic threshold, 3.4 ml O_2/g hr. Dark bars are 12-hr periods of darkness.

In Group 2, measured for 2 weeks from April 30 to May 14, all nine mice showed circadian metabolic rhythms. The mean interval between midpoints of hypometabolic lows was 25.8 hrs (SE $\pm$ 0.77), which is not significantly different from a 24-hour interval. The incidence of hypometabolism was 30.2% the first week and 35.2% the second week. Individual mice varied in their frequency of hypometabolism from one to eight instances during the 2-week period.

For Group 2 as a whole the midpoints of the hypometabolic periods average 1013 hours, and 57.1% of the hypometabolic periods were entirely within the light. However, in the five mice which had sequences of hypometabolic periods, there was a tendency for the midpoints to shift forward in time, as the intervals between midpoints were almost always greater than 24 hours. As a result, 24.4% of the hypometabolic periods had their midpoints within the dark, and 14.3% of them were completely within the dark. Two of these five mice showed a progressive increase in the interval between metabolic lows during the first week, from 25.5 to 33.7 hours and 23.9 to 31.3 hours respectively.

It is clear that Group 2, measured April 30 to May 14, is much more variable than Group 1, measured in early September. Quite possibly this is related to seasonal differences in the metabolic lability of P. longimembris. Individuals that were kept for 9 months in a 10°C room, with food and 12-hour photoperiod, showed the greatest incidence of torpidity in the winter (Chew et al., 1963). The same thing has been found for P. penicillatus and P. baileyi (Hudson, 1964).

In nature, P. longimembris apparently spend long periods underground during the winter, without coming out onto the surface of the ground (Chew and Butterworth, 1964). There is no information on the metabolic condition of the mice during such times. Limited laboratory experiments with P. longimembris kept at 10°C in artificial burrows, show that some mice stay in their burrows for weeks without coming out on the surface. During this time they are not continuously torpid, but arouse every day or so, or remain

normothermic for periods of many days (Chew et al. 1963), Trapping results suggests that during late spring and summer P. longimembris are probably active on the surface every night. During this part of the year they have daily contact with the natural light cycle, and their activity can become entrained by this factor.

By September entrainment can be expected to be maximum. Group 1, measured at this time of year showed metabolic lows (assumed inactivity periods) closely confined to the light period of each day. The mice of Group 2 were measured at a time of the year when, in nature, they would just have ended their period of "winter seclusion", and might be expected to still be relatively labile in their metabolism. Group 2 did have an incidence of hypometabolism twice that of Group 1, and in about 36% of instances the periods of hypometabolism were clearly out of phase with the light period, as much as 180 degrees. In the spring when metabolism is under transition from a highly labile condition to one of stability at a homeothermic level, the sporadic occurrence of torpor may retard the entrainment of activity by photoperiod.

Mice kept in an abnormally warm environment. The mice of Group 3 were kept at an ambient temperature of 35°C, in dry air, and on a 12-hour photoperiod; food and drinking water were supplied in surplus. A temperature of 35°C is near the upper limit of thermoneutrality of P. longimembris (Chew et al. 1963).

These mice showed a pronounced metabolic rhythm, in phase with the photoperiod as for Group 1. There was an average period of 24.1 hrs (SE $\pm$ 1.02) between the midpoints of periods of low metabolism. The form of the metabolic curve was modified by the high environmental temperature. As shown in Figure 2, the metabolic rate usually increased continuously during the dark period. In 35.5% of the cycles the peak metabolism occurred late in the dark period, while in 64.5% it occurred early in the light period. Metabolism decreased abruptly when the lights came on or 1 to 2 hours later. In 89.9% of the cycles the metabolic low occurred in the light, with a mean

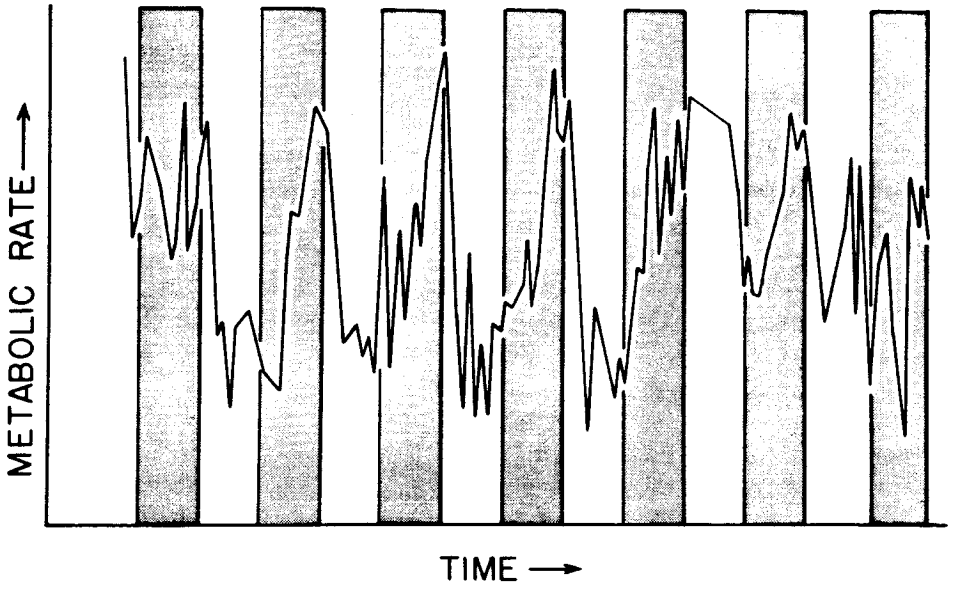

Figure 2. Metabolic rhythm at T_a 35°C. On representative P. longimembris of Group 3. Dark bars are 12-hr periods of darkness.

midpoint of low phases at 1405 hours.

The occurrence of 64.5% of the metabolic peaks early in the light period probably represents a metabolic "overshoot". P. longimembris tends to become hyperthermic at air temperatures of 35°C, especially when the heat production resulting from muscular activity is added to basal heat production. The rise in body temperature would cause a further increase in metabolic rate. Such a self-perpetuating upward trend could cause the metabolic rate to continue to increase for some time after motor activity itself was reduced in response to the beginning of the light phase. In the present experiment it is possible that activity continued part way into the light period; activity per se was not observed or recorded.

Effects of metabolic stress on rhythm.

Deprivation of food. The nine mice of Group 4 were subjected to the same moderate conditions during measurement as Group 2, except they were not given any food. Seven of these mice were the same ones as used in Group 2, 1 week earlier, so that a direct comparison is possible.

There are several obvious contrasts. The mice became hypometabolic every day when they were deprived of food; this is an increase in the incidence of hypometabolism from 32.5% when with food to 100% when without food. When they had food the seven mice had a mean minimum metabolic rate of 0.52 ml O_2/g hr (SE $\pm$ 0.068); without food their mean was 0.32 ml/g hr (SE = 0.041). However, the difference between means is not statistically significant.

When they had food, the mice were hypometabolic principally in the daytime (64.2% of instances). However, when they were without food six of seven mice became hypometabolic during the first period of darkness which began only 3 hours after the start of the experiment (see Figure 3). One mouse remained normometabolic through this night, to become hypometabolic the next day. Quite possibly starvation caused an advance in the occurrence of the first hypometabolic period. The metabolic rhythm then persisted at approximately this new "setting" for the 4 days of the

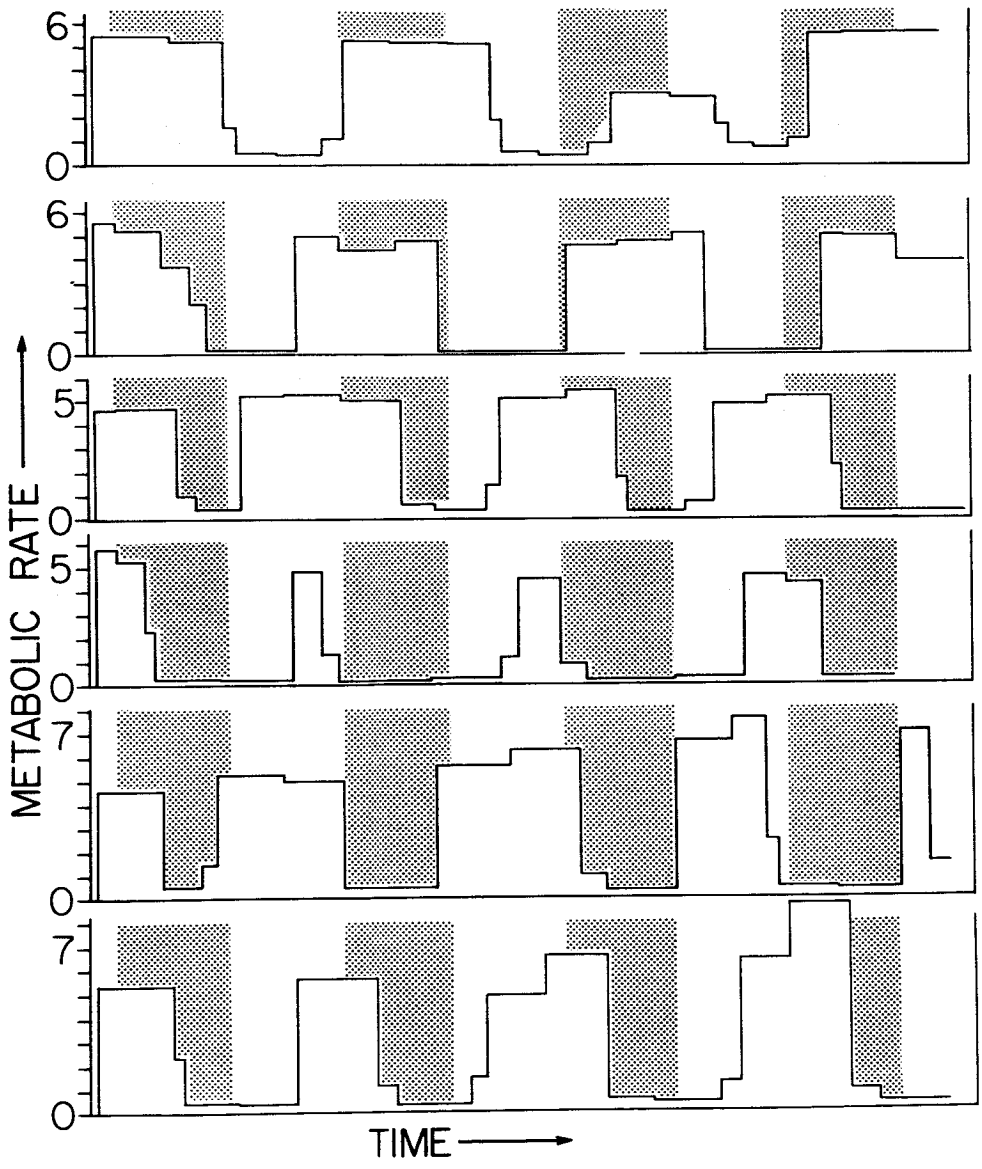

Figure 3. Metabolic rhythms of P. longimembris starved at 22°C. Surviving animals of Group 4. Metabolic rate as ml O_2/g hr; hypometabolic threshold 3.4 ml O_2/g hr. Dark bars are 12-hr periods of darkness.

experiment, 78% of subsequent periods of hypometabolism began in the dark.

In Group 4, the mean interval between midpoints of hypometabolic periods was 24.8 hrs (SE $\pm$ 0.67).

One mouse died without arousing from a second, prolonged hypometabolic period. This animal had the highest initial metabolic rate of all mice in the group. Another mouse died 4 days after the end of the experiment. The facts that this animal had the lowest initial body weight, a high initial metabolic rate, and a delayed entry into its first hypometabolic period, may have contributed to its failure to recover from the stress of the experiment. Similar observations for several other groups suggest that survival and recovery are enhanced by an early entry into a cycle of hypometabolic periods, before excessive use of energy reserves has occurred.

Deprivation of food, plus cold stress. Group 5 was exposed to a double metabolic stress, the lack of food and the low T_a of 10°C. The mice were kept in an atmosphere saturated with water vapor, in order to reduce evaporative water loss, but this had no obvious effect of increasing survival.

In contrast to the mice that were subjected to only food deprivation stress (Group 4), each mouse of Group 5 lost the pattern of daily arousals to a normal metabolic rate and was hypometabolic for more than a day on one or more occasions. The same thing occurred for mice of other groups kept at 10°C without food.

There were some instances when torpor was sustained more than 24 hours at a level of deep hypometabolism (< 0.5 ml O_2/g hr); the mean duration of these periods of "multiple deep hypometabolism" (MDH) are given in Table 1. However, as in Figures 4 and 5, usually the periods of deep hypometabolism were interrupted by periods of moderate hypometabolism (2.0 to 0.5 ml O_2/ g hr). Thus, a circadian rhythm with suppressed amplitude is still discernible. These lesser peaks of metabolism were used in the calculations

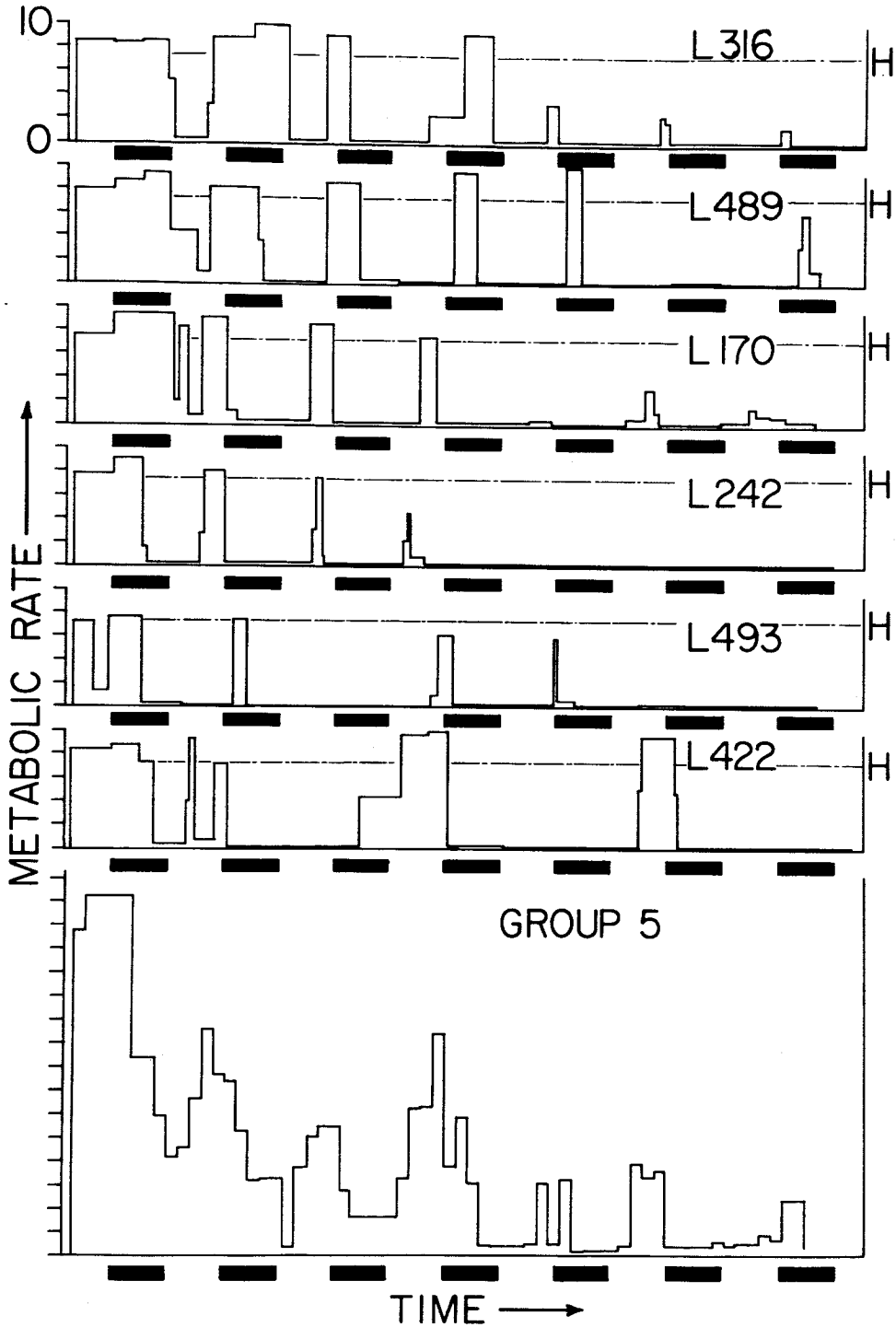

Figure 4. Metabolic rhythms of P. longimembris starved at 10°C. Surviving animals of Group 5; individual graphs above, summation at bottom. Metabolic rate as ml O_2/g hr; H is hypometabolic threshold, 7.3 ml O_2/g hr. Dark bars are 12-hr periods of darkness.

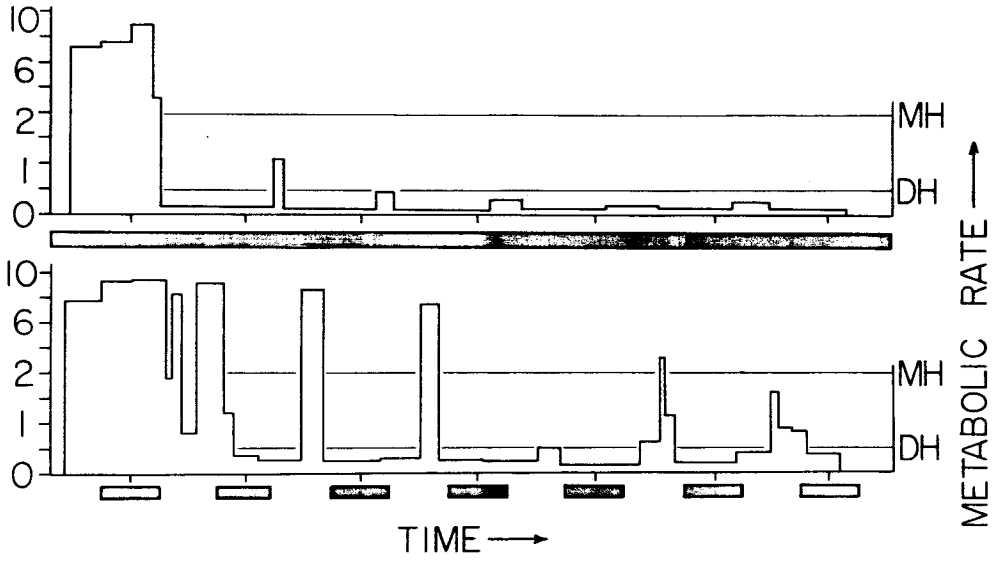

Figure 5. Suppressed metabolic rhythms of P. longimembris starved at 10°C. Two representative animals; upper graph of mouse in continuous darkness, lower of mouse on 12-hr photoperiod.

of periodicities of rhythms, along with peaks of normal metabolism.

In those instances when torpor persisted at the deep hypometabolic level, without discernible fluctuation, it was assumed that there was a fusion of two or more daily periods of deep torpor. For a rhythm with a 24-hour period, the interval between midpoints of hypometabolic periods should be in the sequence of 36, 48, 60, 72 --- hours, as 1, 2, 3, 4 --- arousals are "skipped" respectively. Intervals approximating these durations occur in the measurements for Groups 5, 6 and 7. Rhythm periodicity was estimated in such instances by dividing the observed duration by 1.5, 2.0, 2.5, 3.0 --- respectively.

The mice of Group 5 had a mean interval between midpoints of periods of deep hypometabolism of 23.0 hrs (SE $\pm$ 0.83), if only those periods separated by arousals to a normal rate or to a moderate hypometabolic level are utilized. Using the above method for estimating periodicity during sustained deep torpor, the mice had a mean interval of 23.2 hrs (SE $\pm$ 0.57). Neither maan is significantly different from 24 hours.

The depth of hypometabolism was significantly greater for the mice starving at 10°C than for those at 22°C; mean minimum metabolic rates were 0.12 ml O_2/g hr (SE $\pm$ 0.016) and 0.52 (SE $\pm$ 0.068) respectively.

For Group 5, the discrete periods of normal metabolism are the best basis for analysis of the phasing of the metabolic rhythm with respect to the light rhythm. All six of the mice that survived the experiment entered their first torpor during the last half of the first night, or at the beginning of the next day. For the 17 periods of normal metabolism that occurred following the first torpor: 6 were completely within the 12-hour dark period (as normally expected), 7 started in the light and continued into the dark, and 1 was vice versa. Eleven of the midpoints of the normal metabolic periods were in the dark while six were in the light. There is not a clear entrainment of the metabolic arousals by the photoperiod, but only a tendency for normal metabolism to occur at night, as under moderate conditions. In

Group 5, as in Group 4, the first hypometabolic period may have "reset" the metabolic rhythm, and brought it out of phase with the photoperiod.

Attempts to abolish the metabolic rhythm. Several types of conditions were tested in an effort to bring about sustained deep hypometabolism. These were, in addition to the withdrawal of food: (1) use of "cold-conditioned" animals (Group 6), (2) use of heavy weight animals (Group 7), (3) keeping animals in continuous darkness (Groups 7,8), (4) keeping animals (isolated from external noises) while in continuous darkness (Group 8B), (5) keeping animals in an atmosphere which was 4.5% CO_2 (Group 9).

None of these conditions abolished the stimuli, assumed to be endogenous, which were bringing about arousals from deep hypometabolism. P. longimembris kept for 9 months at 10°C in individual jars with a small amount of nesting material and a surplus of food were frequently torpid but repeatedly aroused. They showed good survival since they were able to feed and thus restore their energy stores during arousals. All the mice that were starved at 10°C (Groups 5, 6, 7, 8) had one or more periods of "multiple deep hypometabolism". The mean durations of these ranged from 42.7 to 56.1 hrs for the different groups; maximum times were 63 to 101 hours. During repeated arousals the starving animals depleted their energy reserves, sometimes to the point of going into prolonged torpor that ended in death (or presumably would have if the experiment had not been terminated.)

Cold-conditioned mice. The mice of group 6 were chosen from those kept for 9 months at 10°C, on the basis of their having the higher incidence of torpidity during the last 4 months (April to July). These cold-conditioned animals had the shortest durations of normal metabolism between periods of hypometabolism, a mean of 2.35 hrs, of all the groups that were compared (5, 6, 7 and 8). The differences are statistically significant.

Heavyweight mice. The mice of Group 7 were the heaviest available from the mice kept at room temperature. They had a mean initial weight of 10.7 g. as compared to 8.3 to 9.5 for Groups 5, 6 and 8. The heavyweight

mice had the longest mean duration of normal metabolic periods, 12.0 hrs, significantly greater than the values for Groups 5 and 6. The heavyweight mice had the best survival of the groups kept at 10°C without food.

Contionuous darkness. Continuous darkness did not significantly alter any of the things that were being measured (see Table 1). If the metabolic rhythm of these mice were truly free running in the dark, i.e., not entrained by some factor other than light, they were not significantly different from a 24-hour rhythm.

The most sensory-deprived group, Group 8b, kept in continuous darkness and isolated from external noises, showed no loss of rhythm. One individual had one of the most precise 24-hour periodicities recorded.

High CO_2 atmosphere. The metabolism of the six mice of Group 9 was measured collectively; the mice were in individual beakers connected in series. The mice were given nesting material, but no food, and were exposed to 4.5% carbon dioxide in dry air. Carbon dioxide has anesthetic properties, and Petter and Mostachfi (1957) speculated that its accumulation in a small nest chamber may induce torpor in the ground squirrel, Spermophilopsis laptodactylus, in nature.

Five of the six mice survived the 6-day exposure. As in Figure 6, a rhythm of arousal and torpor persisted. There was a progressive decline in the heights of the peaks of metabolism. presumably as some animals skipped a particular arousal and/or only partly aroused. A similar decline has been observed in other group experiments with a high oxygen or an air atmosphere. Probably six mice participated in the first recorded arousal, and the equivalent of only one in the last. The first recorded metabolic peak began in the light and continued into the dark, possibly having been shifted by stress. Since the peak-to-peak period averaged 22.5 hrs, the metabolic peaks occurred progressively earlier in the light phase.

In Figure 6 the sharpness of the metabolic peaks indicates a high degree of synchrony of the rhythms of individual mice; a secondary peak can

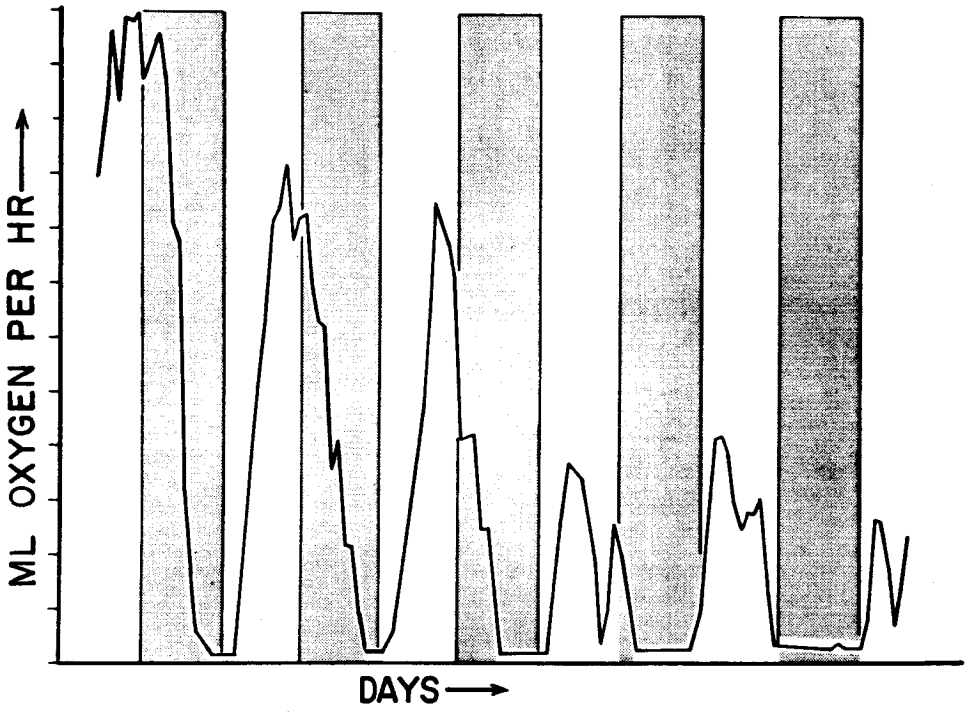

Figure 6. Metabolic rhythm of a group of six P. longimembris, starved at 10°C. Mice of Group 9: 12-hr photoperiod, in air with 4.5% CO_2,

be interpreted from the fourth day onward, with a period of 21.5 hrs. Synchrony to a lesser degree is present in the summated results of the individually measured mice of Group 5 (see Figure 4). Another group experiment, with six mice in separate small compartments of the same container, showed considerable asynchrony of peaks.

Effect of irradiation. The mice of Group 10 were measured under the same moderate conditions as Group 2 (which serves as a control) except they were given an acute whole body gamma radiation exposure of 1400 rads from a cobalt 60 source just prior to the beginning of the metabolic measurements. The mice were measured for 6.5 days, removed for weighing, and then returned for a further 7-day period of measurement. P. longimembris has an LD 50 at 30 days of 1510 rads of ionizing radiation in comparison to 628 rads for CF1 white mice (Gambino and Lindberg, 1963).

The damage caused by the radiation did not significantly change the periodicity of the metabolic rhythm. The mean interval between midpoints of hypometabolic periods was 24.0 hrs (SE $\pm$ 0.33) for the irradiated mice (Group 10) and 25.8 (SE $\pm$ 0.77) for the control (Group 2).

The irradiated mice did show a greater incidence of hypometabolism than the controls, 53.1% versus 32.5%. This may be the result of radiation damage, or it may be chance variation. Individual mice of Group 10 varied from 0% to 100% incidence. The majority of the hypometabolic periods of the irradiated mice began in the dark and ended in the light (54%), while for the controls the majority was entirely within the light (57%). Some of the irradiated mice showed a shift of their rhythm after being disturbed for weighing on the 7th day. The hypometabolic midpoints were an average of 2.76 hrs later the second week. No shift of this magnitude was observed for Groups 2 and 11, which were similarly weighed in the middle of a 2-week measurement period.

Mice of Group 11, irradiated with 1400 rads and then kept at 35°C, are directly comparable with the control Group 3, measured just previously. All mice survived the first week after irradiation; four mice died after 8 or 9

days. The total group for the first week (Group 11a) and the four mice that survived for 4 weeks (Group 11b) had the same rhythm as the controls. The irradiated mice did have higher maximum metabolic rates, a mean maximum of 5.19 ml O_2/g hr (SE $\pm$ 0.15) for Group 11b versus 4.45 ml O_2/g hr (SE $\pm$ 0.087) for the controls.

Comparison of species of Perognathus. Three species, P. longimembris, inornatus, and formosus were compared in a series of experiments under the same conditions: 10°C, dry air, continuous darkness, no food. Certain of the results are compared in Table 2. There are no significant differences in the periods of the metabolic rhythms; all are circadian.

The results suggest that the larger species have less metabolic lability. There is an inverse relationship between mean body weight and the incidence of hypometabolic periods. The smallest species, P. longimembris showed five instances in which individuals remained hypometabolic for more than 24 hours and then spontaneously aroused. The larger species had no instances of sustained torpor, except those which terminated in death.

Tolerance for hypometabolism at T_a 10°C also may vary inversely with body weight. Six of eight P. formosus went into a sustained torpor and died after periods of 3.8 to 5.7 days. Of the two that survived, one showed a consistent circadian rhythm of deep torpor and arousal, while the other remained normometabolic. Tucker (1962) observed that P. californicus (Avg. Wt. = 20.9) cannot arouse spontaneously from body temperatures below 15°C. In the closely related genus, Dipodomys, D. merriami cannot recover from body temperatures below 12 to 15°C (Lipp and Folk, 1960).

The poor survival of P. longimembris at 10°C in some experiments suggests that 10°C is near the arousal threshold for the species. The present experiments are not a fair test however, of what the pocket mice can do if they have food available during arousals.

Another group of P. inornatus was kept without food at 10°C, in continuous darkness, in air saturated with water vapor. The mice were kept

Table 2. Comparison of the metabolic rhythms of P. longimembris, inoratus, and formosus. All groups measured at 10°C, without food, in continuous darkness, and in dry air.

Species	Mean Weight, g	Rhythm period[b] mean + SE, hrs	Duration of MDH[c], hrs	Incidence of deep hypo-metabolism
longimembris n = 7/8[a]	10.7 (10.5-11.7)	23.7 ± 0.79	47.9 (n=5)	100%
inornatus n = 4/4[d]	14.6 (13.0-17.4)	24.8 ± 0.75	(n=0)	83.3%
formosus n = 2/8	20.5 (16.9-28.2)	25.0 ± 0.76	(n=0)	61.1%

a - surviving number/initial number.

b - a mean interval between midpoints of hypometabolic periods.

c - MDH, multiple deep hypometabolic periods persisting more than 24 hours.

d - one animal died 2 days after end of experiment, without arousing from torpor.

in individual chambers, connected in series, and were given nesting material. As in Figure 7, the record can be analyzed as a rhythm with 21.0 to 25.0 hour intervals (mean 23.2 hrs). After the third day a lessening of the synchronization of animals disrupted the earlier clear cut rhythm. There was a 100% incidence of deep hypometabolism. As with P. longimembris groups, there was a progressive decline in the metabolic peak each day when some animals failed to arouse daily.

The adaptive values of a persisting 24-hour metabolic rhythm. The presence of a persistent rhythm which brings about arousals from torpor on a circadian period or multiple thereof can have both physiological and behavioral advantages.

Periodic arousals can insure the correction of physiological deviations that occur during torpor. The sequence of torpor, internal change, arousal, restoration of change and re-entry into torpor, could be a simple feedback relationship.

The records of suppressed circadian metabolic cycles of P. longimembris undergoing sustained torpor (see Figures 4 and 5) directly support the hypothesis of Folk (1957) and Folk et al. (1958) that: (1) certain species of hibernators have rhythms of deep and shallow hibernation, (2) when an arousal-stimulus coincides with the shallow phase, the animal may arouse, (3) if the metabolic rhythm is entrained by photoperiod before hibernation begins, then the animal will tend to arouse to normal activity during that part of the diel environmental cycle to which it is adapted. Folk and associates found circumstantial evidence for this for the ground squirrel, Citellus tridecemlineatus. Records in Pohl (1961) show the same thing for the metabolism of hibernating dormice, Glis glis.

Since P. longimembris take 4 to 7 days to shift their motor activity pattern back into phase with a reversed light-dark cycle in laboratory experiments (R. Lindberg and P. Hayden, personal communication) pocket mice in nature that happen to arouse during the night could take the same time. In laboratory experiments, motor activity persists in "inappropriate"

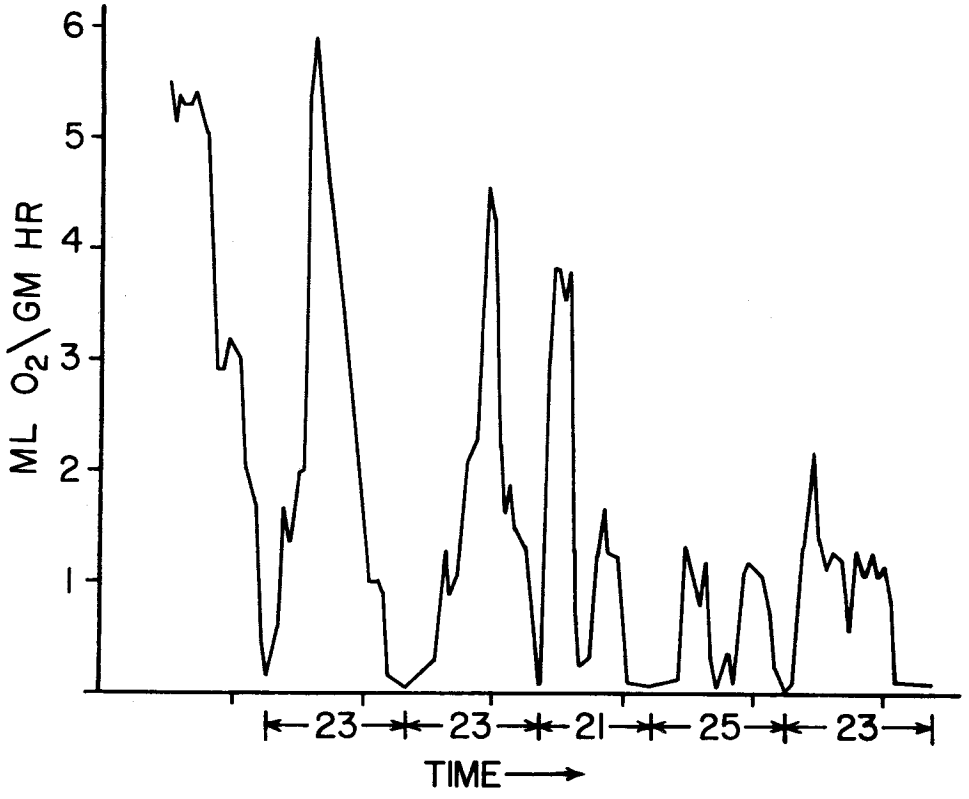

Figure 7. Metabolic rhythm of group of six P. inornatus starved at 10°C. Mice in continuous darkness, in air saturated with water vapor, Feb. 20-26, 1963.

lighting situations until the phase shift of activity is completed. If the same thing were to occur in nature the disadvantage to the individual could be fatal.

The present study strongly indicates that metabolic stress (from starvation, cold exposure) can throw off the synchronization of metabolism and photoperiod. For example, the "unstressed" Groups 1 and 3 had midpoints of activity periods of 0102 and 0203 hours respectively (normal), while the stressed Groups 4, 5, 6 and 7, had activity midpoints of 1901, 1745, 1816 and 2080 hours (abnormal in that much of activity occurred before beginning of darkness). Most of the shift can be attributed to an "advance" in the occurrence of the first hypometabolic period by about 6 hours which "sets" the "clock of activity" forward by this amount. Folk (1958) found a similar shifting of activity peaks by 6 to 10 hours in Citellus tridecemlineatus exposed to cold.

Such an effect of stress could cause difficulties in nature. However, such abrupt stressing, particularly by starvation, is probably very uncommon in nature. When a stress is gradually applied, as with the gradual decline in food abundance in nature, correct phasing may be retained; obviously, this remains to be studied. A lack of synchronization in the wintertime would not be critical if P. longimembris remain in their burrows during this time, as seems to be true in some parts of their range.

The present study also suggests that synchronization of behavior and lighting may be impaired in the springtime, when pocket mice may be changing from a metabolically labile condition to one of relative stability. This could pose a serious difficulty in nature.

Although it would be theoretically advantageous for a pocket mouse to be able to go into a sustained deep torpor when it was starving, and thus prolong its survival, P. longimembris has not yet been experimentally induced to do so, for more than 4.2 days in one instance. Considering its ecology which is adapted to chronic shortages but not to complete absences of food, this species cannot logically be expected to have a mechanism which

can forecast the improbability of the early reavailability of food, and call into play a prolonged, unbroken hibernation. Judging from the work of Morrison (1960), the body size and metabolic characteristics of P. longimembris are such that probably it is incapable of long term torpidity from one "good" season to the next. So, any situation in nature in which food is completely lacking, which the pocket mouse does not meet by moving to another area, will probably be fatal.

The pattern of arousal from torpor on a 24-hour rhythm (or some small multiple of 24 hours) does allow effective conservation of a minimal food supply. For example, the mice of Group 7 were deeply hypometabolic for 78.0% of the experimental period, moderately hypometabolic for 2.9%, and normally metabolic for 19.1% of the time. The hypometabolism resulted in a saving of 80.4% on the estimated metabolic cost of maintaining normal metabolic rates for the entire time.

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FREE RUNNING PERIOD AND PHASE SHIFTS IN TWO SPECIES OF POCKET MOUSE

P. Hayden

INTRODUCTION

The existence of a circadian rhythm in the genus Perognathus has been well documented (1,2,). The purpose of the present effort was to determine the persistence and stability of the rhythm as a function of photoperiod and time. Two studies were initiated; one at Northrop Space Laboratories, reported in this paper, and another at Princeton University which will be reported at a later time. Both studies focused on the definition of a free running period in Perognathus longimembris although some attention was also given to Perognathus formosus. In the NSL studies, continuous measurement of oxygen consumption and locomotory activity were used to define periodicities while the Princeton studies continuously measured body temperature with an implanted temperature telemeter developed by NSL.

METHOD

Measurement of Locomotory Activity

Gross motor activity was used in preference to wheel-running activity because of space limitations during oxygen consumption measurements and the limited nature of wheel-running activity by pocket mice. Although there are several ways of measuring gross motor activity (chamber on tambours, springs, rotating peg, etc.) each presented special problems with the pocket mouse. The electrical recording of gross motor activity via the stimulation of a piezo-electric transducer had been attempted previously with marginal success.

However, the data of Gans and Bonin (3) on subterranean burrowing of worm lizards encouraged further development of a simplified modification to be used on pocket mice.

The activity monitoring system consists of three major units: a) the animal housing chamber, b) the amplifier, c) graphic recorder. The animal chamber is a polystyrene dish (8" dia. x 3" high) with a perforated cover. Each animal is provided with sand and sufficient food for the experimental period. The piezo-electric transducer (a musical contact microphone) is cemented directly to the bottom of the animal chamber. The movement of the sand by the animal triggers the transducer. The output from the transducer is fed into an amplifier powered by a six volt battery. The six transistor amplifier has a "flip-flop" circuit which controls the operation of a sensitive relay.

A multi-channel events recorder (Esterline-Angus, 20 channel, Model A620T with temp-pens) is used to record the actuation of the relay. The paper speed is slow enough (1.5 inches/hour) so that periods of intense activity are recorded as a nearly solid black band instead of individual events.

Measurement of Oxygen Consumption

The method of continuously monitoring oxygen has been described in detail elsewhere (2). Briefly, the animal is confined in a closed system (Metabolor) submerged in a constant temperature water bath and attached to a recirculating pumping system with appropriate filters for removal of carbon dioxide and water vapor. As the entire system undergoes pressure changes (oxygen decrease, removal of CO_2 and H_2O) sensing manometers detect the change and replenish the oxygen by activating solenoid valves. As the oxygen is replaced in the system, a graphic record is made on a time chart. Knowing the volume replaced and the time between replacements, oxygen consumption per unit time is calculated.

When gross motor activity was recorded concurrently with oxygen consumption, a metal diaphram, with the microphone attached, was fitted into the bottom of the chamber that normally served in oxygen utilization experiments. Both of these measurements were recorded on different channels of the same recorder.

Animal Housing

During experiments the animals were normally housed in either the plastic chamber (locomotory activity) or 1000 ml tall form beakers (oxygen consumption and activity), each provided with sand and food. In one experiment (Expt. 6, Table 1) individual animals were housed in metal mesh tubes covered with asbestos and inserted into a 1-1/2 I.D. aluminum tube.

Experiments involving measurement of oxygen utilization were done in the Metabolor while experiments involving only measurement of locomotory activity were done in a constant temperature room. All experiments were conducted at 21°C.

Summary of Experiments

Table 1 summarizes three categories of experiments which describe metabolic and locomotory periods in response to;(a) a twelve-hour light, twelve-hour dark light regime, (b) changing light regimes (Phase Shift), and, (c) constant dark or constant light (free-running period). The free-running period reflects the inherent biological rhythm expressed by the animal in a constant environment without the entraining influence of a photoperiod.

Table 1. Summary of circadian periodicity experiments on pocket mice

Expt. No.	Species & Date	No. Animals	Kind of Measurements Taken	Photoperiod	Duration
1.	P. longimembris March 11-26	6	Activity and Oxygen Consumption	12L - 12D	14 Days
2.	P. longimembris March 27 - April 24	8	Activity and Oxygen Consumption	12L - 12D 24D 24L	7 Days 10 Days 10 Days } 27 Days
3.	P. longimembris May 5 - 20	8	Activity (Phase-Shift)	12L - 12D 12D - 12L	8 Days 7 Days } 15 Days
4.	P. longimembris Aug. 1 - 25	16	Activity (Free-running Period)	24D	24 Days
5.	P. longimembris Aug. 26 - Sept. 18	16	Activity (Free-running Period)	24D	22 Days
6.	P. longimembris Oct. 13 - Nov. 5	8	Oxygen Consumption (Free-running Period)	24D	22 Days
7.	P. formosus May 27 - June 6	13	Activity	12L - 12D	9 Days
8.	P. formosus Sept. 18 - Oct. 9	16	Activity (Free-running Period)	24D	21 Days

RESULTS

Experiment 1 (Table 1) was orignially scheduled to run for 10 days but was extended to fourteen days when all components were observed to be functioning well. As a result of this extension the food supply ran out inducing hypometabolism for a longer period during the last three days of the experiment (indicated by black lines Fig. 1). In five of the six animals studied the data show that the onset of hypometabolism occurred generally in the early morning lasting until the early afternoon. However, one animal of this group showed a distinct rhythm of arousal from hypometabolism for a few hours every afternoon (Fig. 2). Apparently it maintained normal body temperature only 4.5 to 6 hours (16%-25%) of the day. At the termination of the experiment this animal was found to have over half of its food remaining. The activity record for this animal is virtually blank except for the afternoon normometabolic period.

The metabolic periodicity of this animal seems to experience a phase-shift (as measured by midpoints of normometabolic periods) during the early part of the record. From the active period of day 3 to day 9 there is a decrease of 30 mins/day (a period of 23 hrs. and 30 mins.). If the experiment had been terminated at 10 days, it would have appeared that this mouse had a "free-running" period. From day 10 until day 15 the midpoint stabilized around 1630 hrs. with no greater deviation than 23 mins. The time of entry into hypometabolism during days 10-15 was well defined at 1915 hrs., with a maximum variation of about 15 mins.

Of 46 period lengths determined between midpoints of hypometabolic states of individuals in this group, 61% were between 23 and 25 hrs. If average period lengths for individuals are considered, 4 of the 6 were between 23 hrs. 45 mins. and 24 hrs. 15 mins. The overall average for the groups is 23 hrs. 56 mins.

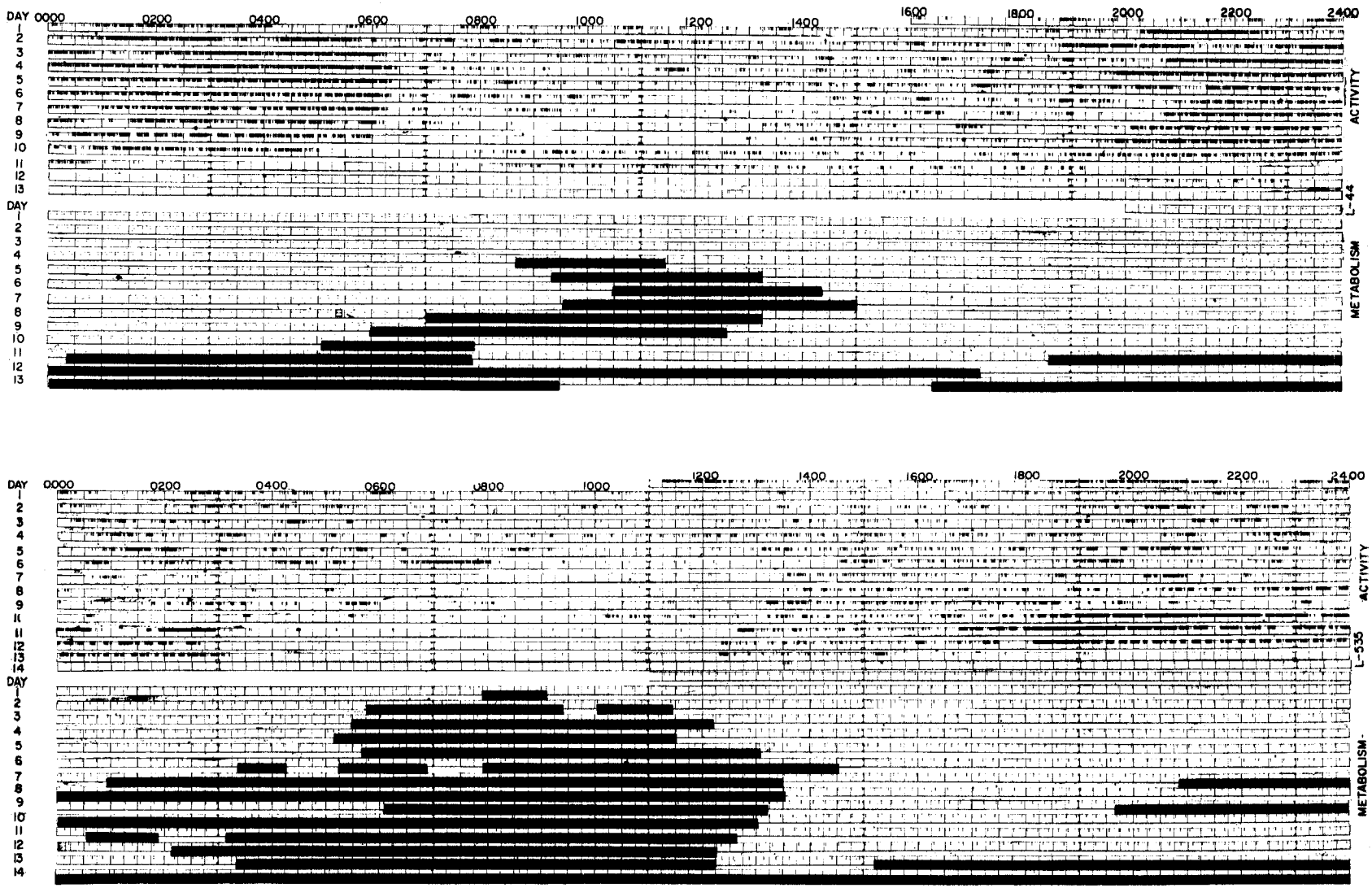

Figure 1 Gross motor activity and oxygen consumption records of two pocket mice (P. longimembris) maintained in the metabolor for a period of two weeks at 21°C with a photoperiod of 0600-1800 hr. light. The hypometabolic periods are indicated in black.

Perognathus Longimembris - Gross Motor Activity and Oxygen Consumption - Metabolor, Air Atmos., 21°C, Food, 12L-12D (0600 - 1800 hrs. Light)

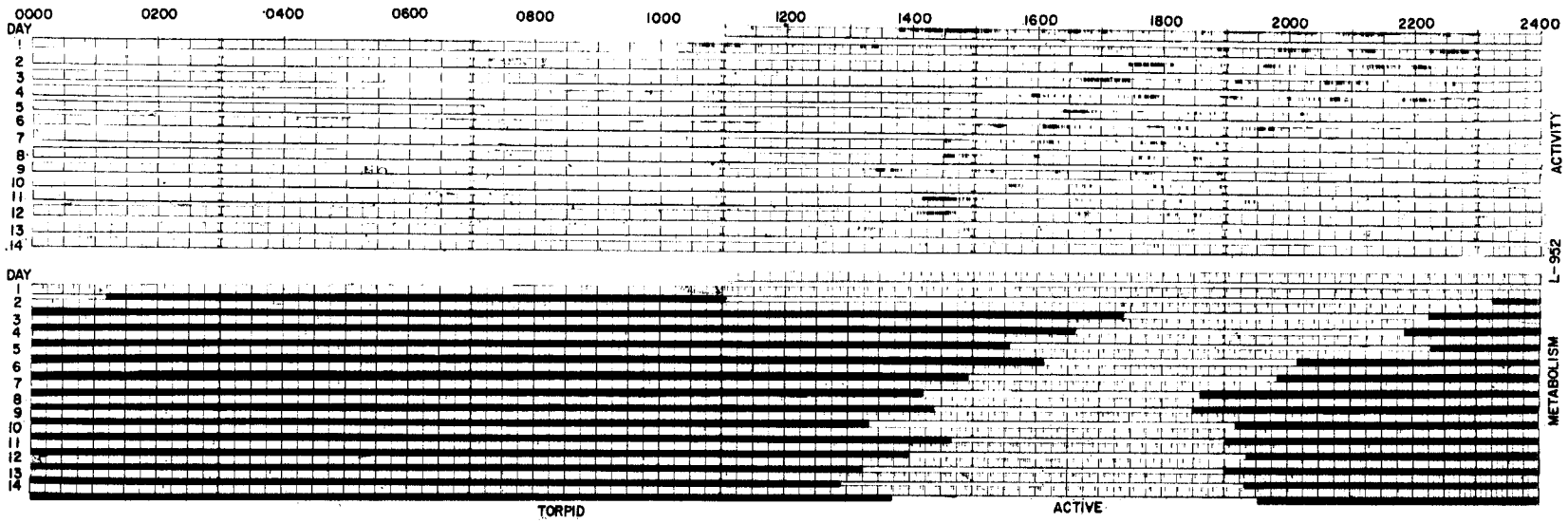

Figure 2 Gross motor activity and oxygen consumption records of a pocket mouse (P. longimembris) maintained for two weeks at 21°C in the metabolor. The dark areas are periods of hypometabolism.

Perognathus Longimembris - Gross Motor Activity and Oxygen Consumption - Metabolor, Air Atmos., 21°C, Food.

Without exception, the data derived from the gross motor activity was a mirror image of oxygen consumption. While this coupling is not unexpected the two kinds of measurements are not necessarily redundant.

As to be expected, the motor activity pattern was strongly influenced by light (4,5,6). In general, if activity had not stopped before lights-on (0600), it was sharply curtailed thereafter (Figure 1, top). Intense evening activity took place several hours after lights out (~ 2000 hrs.).

Experiment 2 (Table 1) tested the effect of various light regimes on activity and periodic hypometabolism in a 27 day experiment. The animals were placed in a 12L-12D light regime for 7 days, then in total darkness (24D) for 10 days, and finally in constant light (24L) for 9 days.

Two animals showed no hypometabolism during the experiment while the six others showed torpor which varied from occasional to nearly everyday. Figure 3 is an example of the two extremes in kinds of measurement. The top animal had an almost perfect hypometabolic record with diffuse activity, while the bottom animal was the reverse. In the hypometabolic animal a stabilized torpid period was established between about 0430 hrs. to about 1300 hrs. during the 12L-12D regime. The animal responded to the 24D regime by a general shift of the hypometabolic period to an earlier time (a period of 23 hrs. 19 min., determined from midpoint of hypometabolic state). With the onset of continuous light the onset of the torpid state was shifted forward in time so that a period of 24 hrs. 53 min. is achieved (midpoints of hypometabolic state).

The gross motor activity (Figure 3, bottom) gives data that is not so clear cut as metabolism although the trends can be found. In this particular animal the cessation of activity seems to be the strongest guide point as to its periodicity. Under a 12L-12D light regime the major portion of activity occurred between 1600 and 2000 hrs., with a shift indicated to the later time. With the beginning of 24D, the later period of activity was maintained but with intense activity occurring earlier in the day. A period

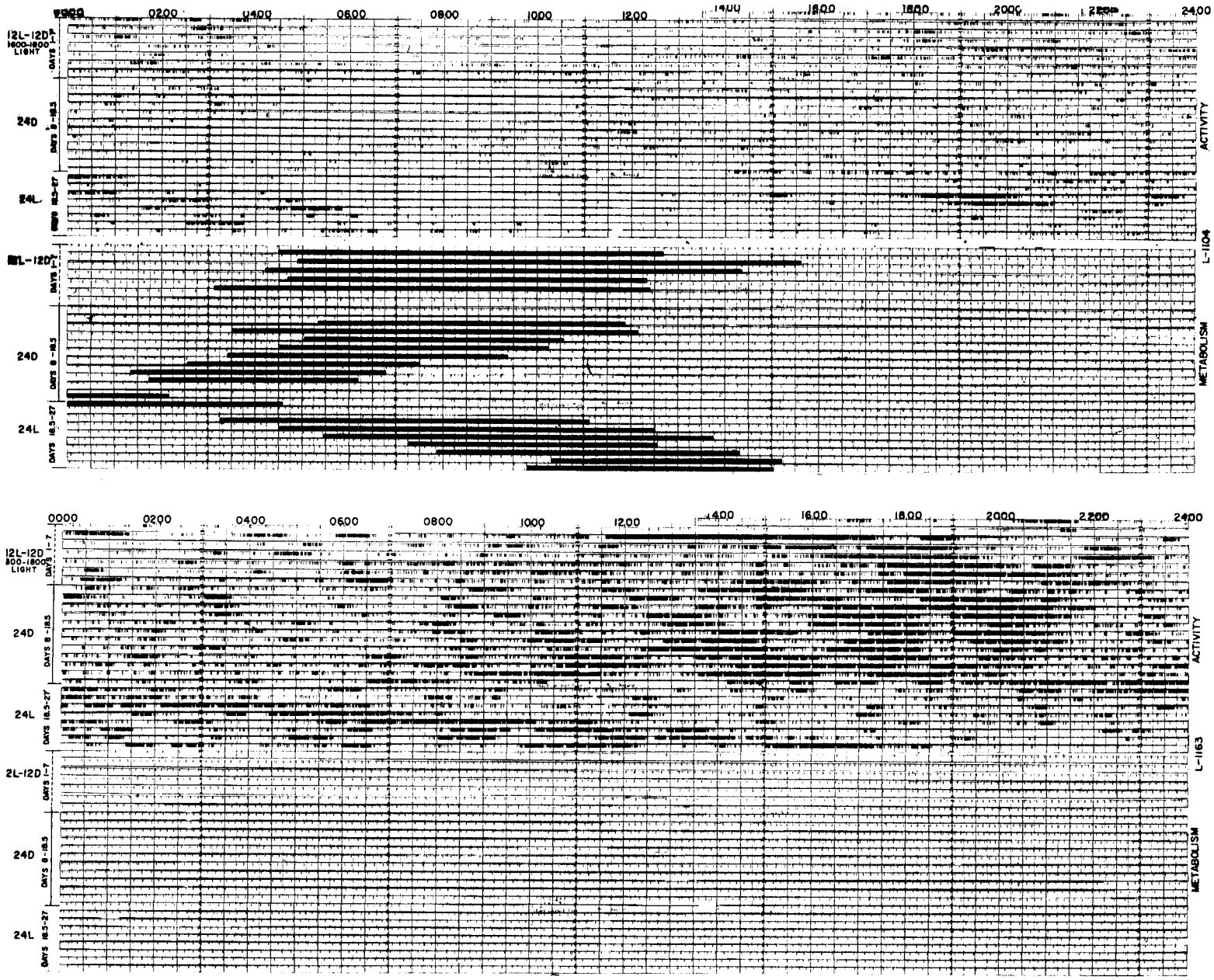

Figure 3 Gross motor activity and oxygen consumption records of two pocket mice (P. longimembris) maintained in the metabolor at 21°C and exposed to three different lighting regimens (normal, constantly dark, constantly light).

Perognathus Longimembris - Gross Motor Activity and Oxygen Consumption - Metabolor, Air Atmos., 21°C, Food, Various Light Regimens

of 23 hrs. 45 mins. is indicated by cessation of activity during this light regime.

A radical change occurs during the continuous light portion of the experiment. The afternoon-evening pattern is no longer there, and a fast changing pattern with a period of 25 hrs. 52 mins. is evident.

The frequency of period lengths (as determined by midpoints of hypometabolism and/or onset or cessation of activity) is present in Figure 4. Data for this figure were derived from only five animals (Expt. 2, Table 1) as one died because of a mechanical malfunction and two showed no hypometabolic periods. During the continuous light regime, few periods of hypometabolism were observed so that the number of period lengths determined by that method are less. Of the 116 individual period lengths determined during the three lighting regimes a grand average of 24 hrs. 1 min. (12L-12D), 23 hrs. 49 min. (24D) and 25 hrs. 57 min. (24L) was established. This is in agreement with the hypothesis that a nocturnal animal will have a shorter period in constant dark and a longer period in constant light.

Experiment 3 (Table 1) investigated the ability of the little pocket mouse to readjust to the 180° reversal of the light regime by monitoring gross motor activity. Each individual chamber in the light-proof walk-in constant temperature box was fitted with 6W incandescent bulbs. The bulbs were controlled to 8 candle power falling on the floor of the chamber (Weston photoelectric meter). The animals were exposed for 8 days to a 12L-12D light regime (normal lighting schedule in holding facilities).

The lighting schedule was then reversed for the remainder of the period. The activity data from four individual mice are presented in Figure 5. All animals completed the shift in 7 days or less. This kind of shift time has been observed by other investigators (4,5,6). Animal L-630 (Figure 5) completed the light rephasing in 4 days. This was accomplished in two large forward steps of about 5 hours each. The lights-on in the evening of the phase reversal apparently completely inhibited the evening activity period. This kind of initial inhibition is noted in all cases.

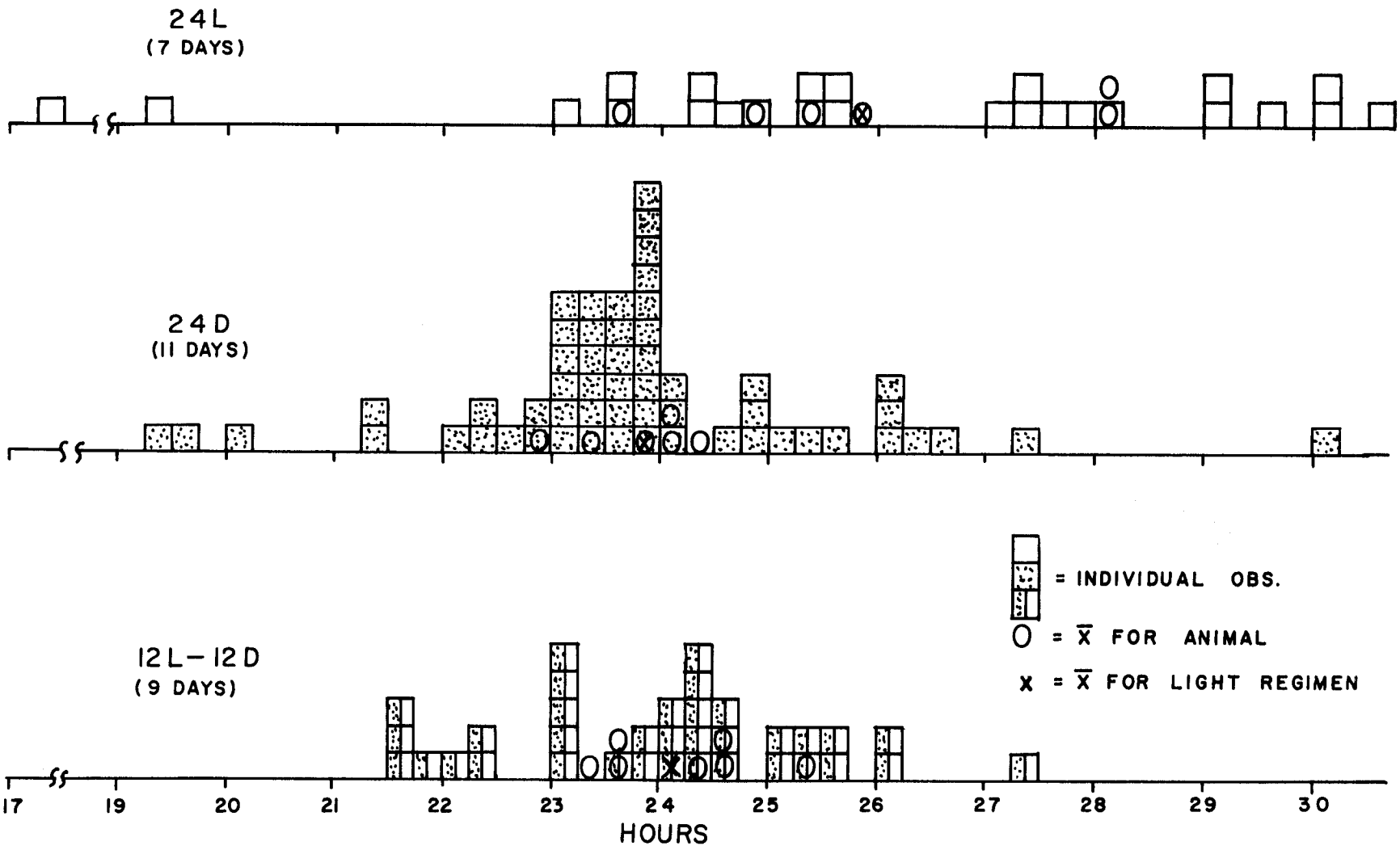

Figure 4 A comparison of period lengths of P. longimembris as determined in the three light regimens (normal, constantly dark, constantly light).

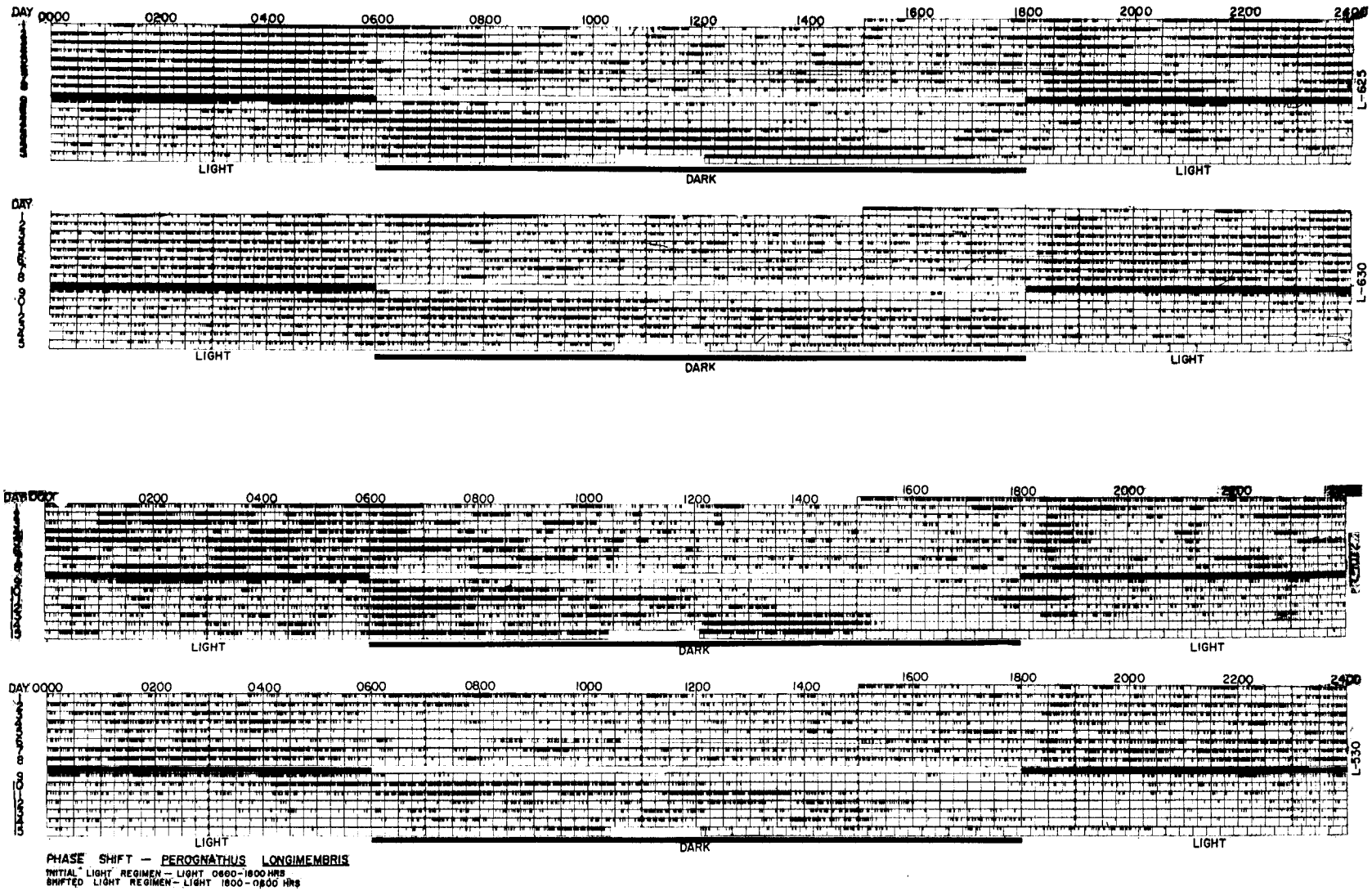

Figure 5 Gross motor activity of four pocket mice (P. longimembris) exposed to a normal photoperiod (0600-1800 light), and the 180° reverse of this photo-period (1800-0600 light). The reversal of lighting occurred at 1200 hrs. on day 9.

Experiment 4 and 5 (Table 1) was designed to determine the free-running period of pocket mice by recording gross motor activity in constant dark.

The free-running period was determined by fitting a straight line to the data, calculating an overall time shift, and expressing the shift as a shortening of the period per day (Figure 6a is representative of both experiments). Table 2 is a tabulation of free-running periods grouped into 15 minute intervals and expressed in per cent of total mice showing a particular period in both experiments.

Table 2

A summary of free-running periods of pocket mice (Perognathus longimembris) as determined from analysis of gross motor activity

21 hrs. 0 21 hrs. 15 min	21 hrs. 45 min. 22 hrs. 00 min.	22 hrs. 30 min. 22 hrs. 45 min.	22 hrs. 45 min. 23 hrs. 0 min.
4% (1)	4% (1)	12% (3)	12% (3)
23 hrs. 0 min. 23 hrs. 15 min.	23 hrs. 15 min. 23 hrs. 30 min.	23 hrs. 30 min. 23 hrs. 45 min.	23 hr. 45 min. 24 hr. 0 min.
20% (5)	16% (4)	16% (4)	16% (4)

These values are in agreement for free-running periods determined in Peromyscus (7).

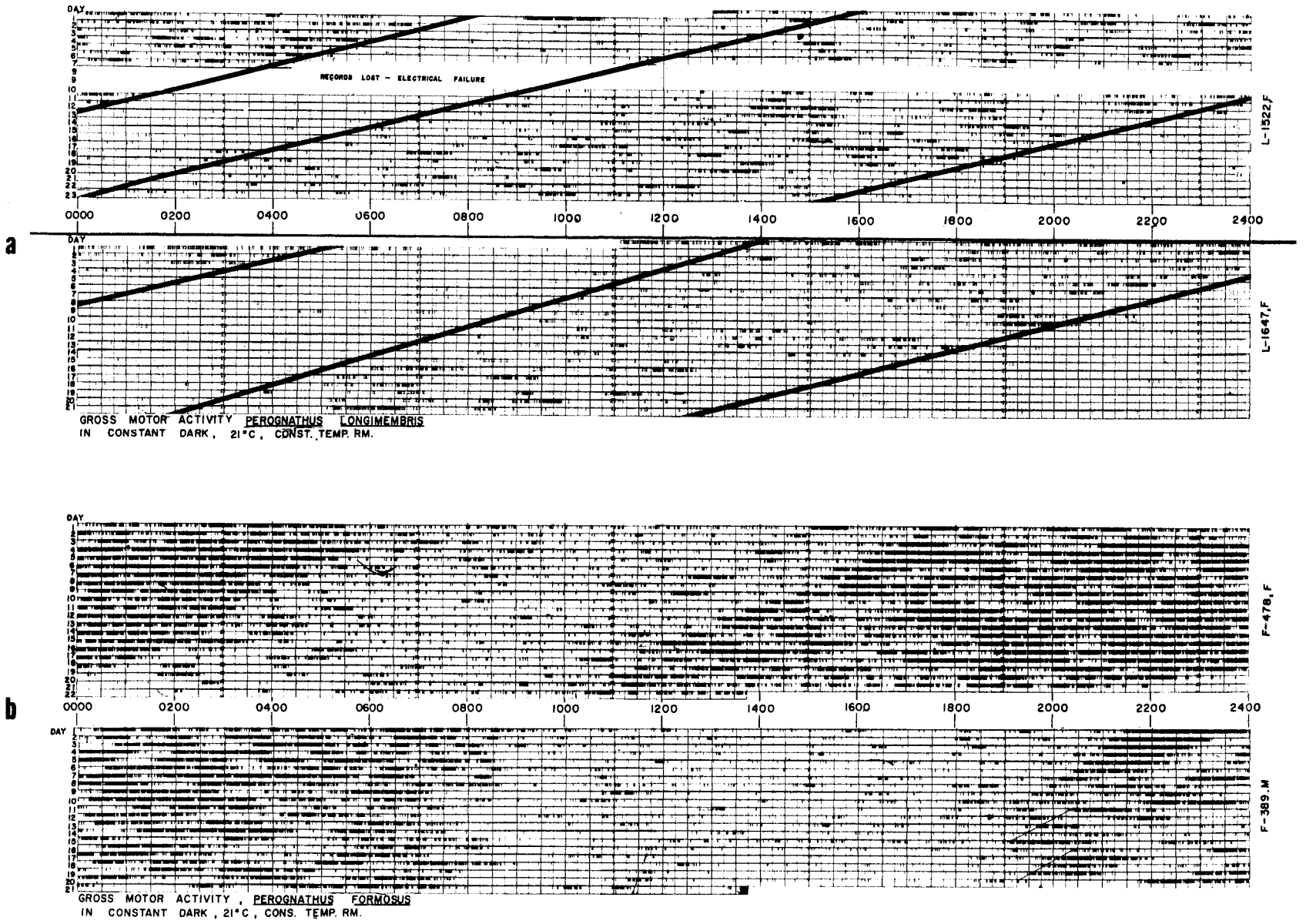

Figure 6a and 6b Gross motor activity of pocket mice (P. longimembris and P. formosus) maintained in a constant temperature box at 21°C and in constant dark. Activity periods are delineated by the heavy black lines.

Experiment 6 (Table 1) was undertaken to detect any effects upon the expression of daily hypometabolic periods resulting from confining the animals in simulated space flight hardware. It was suspected that removal of the substrate dusting material (sand), and closer confinement in a tubular structure would affect the periodicity. The experimental chambers consisted of three tubes 9" in length, three tubes 15" in length, and two pint jars with sand substrate as controls. Only oxygen consumption data was collected and reduced to ml of oxygen consumed per hour per gram mouse (STP). These values are plotted in Figure 7. This figure adequately demonstrates the daily free-running hypometabolic state that appears to repeatedly move across the 24 hr. "window" of observation.

Of the eight animals, two in bottles and one in a 15" tube demonstrated good circadian periodicity. A free-running period of 22 hrs. 45 min., 23 hrs. 25 min., 23 hrs. 30 min., was calculated for these animals.

The animals in the short (9 in.) tubes showed a suppression of the hypometabolic state. Each had a long hypometabolic period (9-12 hrs.) the first several days, but was progressively shortened until it disappeared on the 8-10 day.

Experiment 7 and 8 (Table 1) measured environment and free-running period in constant dark on another species of pocket mouse, Perognathus formosus (long-tailed pocket mouse).

Representative data is not presented, but all records showed a strong entrainment to light.

In the determination of a free-running period (Expt. 8) for this species several interesting records were obtained. One animal was observed to have a period of greater than 24 hrs. (24 hrs. 20 min.).

In one animal (Figure 6b) the onset of evening activity presented a different picture from any animal observed. The first 10 days were

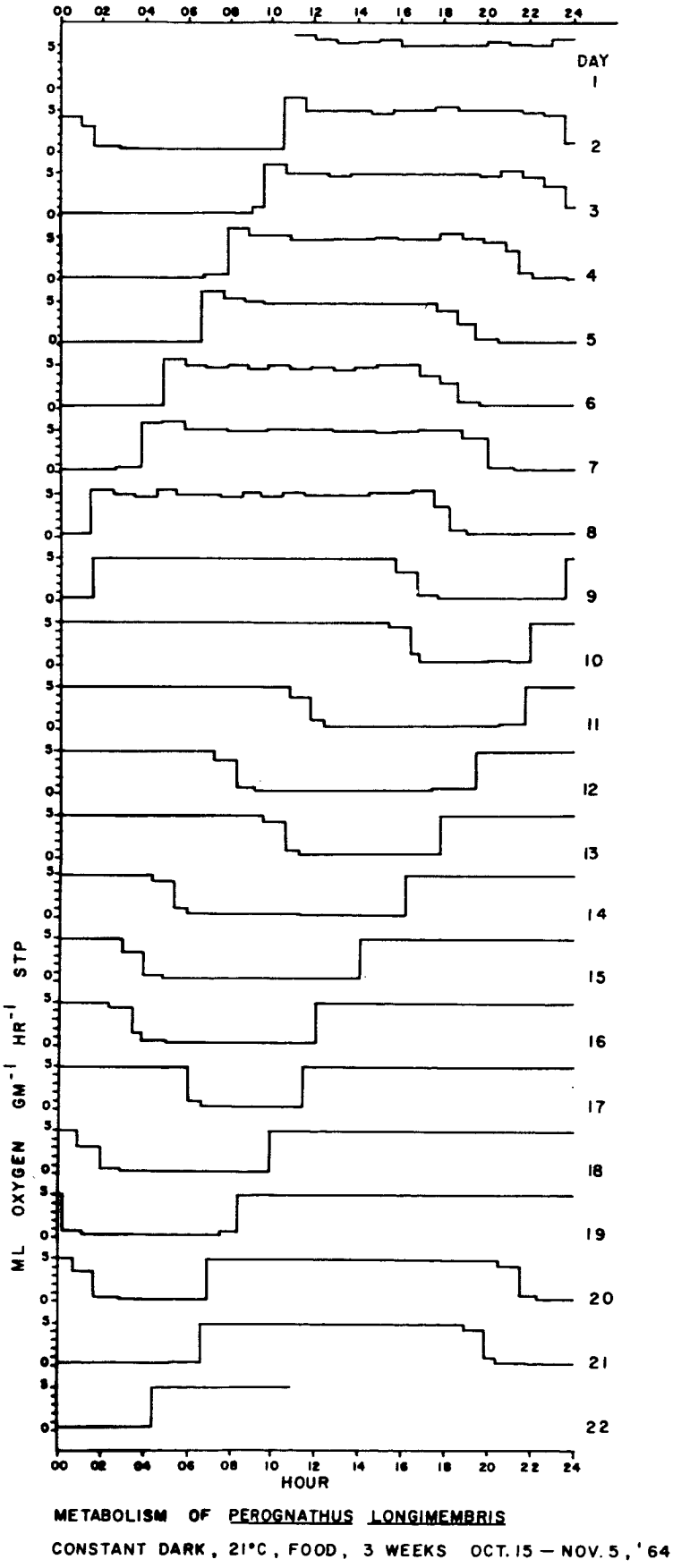

Figure 7 Metabolism of a P. longimembris maintained in simulated space hardware in metabolor for three weeks at 21°C, constant dark. Actual values of oxygen consumption during normometabolic periods are plotted from day 1-9. Days 10-22 normometabolic values are averages for that period.

characterized by a 15 min/day increase in the time of onset of major activity. On the eleventh day there was a 1-1/4 hr. set-back. The 15 min/day increase is evident for 4 more days, when another set-back of 1-1/4 hrs. takes place. Unfortunately, another set-back was not observed as the experiment was terminated the night it probably would have occurred.

In effect, it would appear that the animal could maintain a near 24 hour period by correcting a 15 min/day "drift" by a major shift every several days. This resetting was observed only in the onset of major activity. The cessation occurred at a fairly constant time.

The free-running period values for P. formosus are given in Table 3.

Table 3

Free-running period length of P. formosus as determined from gross motor activity measurements

21 hrs.45 min.	22 hrs.50 min.	23 hrs.10 min.	24 hrs.00 min. 23 hrs.40 min.	24 hrs.20 min.
8.3% (1)	8.3% (1)	8.3% (1)	66.8% (8)	8.3% (1)

SUMMARY

1. A simple device was assembled to continuously measure gross motor activity of pocket mice under normal housing conditions.
2. With a 12L-12D photoperiod both species maintain a well regulated sequence of quescence vs. activity (entrained to photoperiod).
3. P. longimembris can complete a 180° shift in the light regime in 4-7 days.
4. P. longimembris responds to various light regimens (24D, 24L as typical nocturnal animals by lengthening its period in constant light and shortening it in constant dark.
5. A free-running period of from 21 hrs. to near 24 hrs. was documented, with 68% observed to be between 23 hrs. and 24 hrs.
6. A near perfect metabolic circadian rhythm was demonstrated in which oxygen consumption dropped drastically. The endogenous "clock" apparently worked accurately with daily body temperature fluctuations of about 15°C.
7. The long-tailed pocket mouse (P. formosus) was determined to have a free-running period essentially the same as the little pocket mouse (P. longimembris), except one case in which it was greater than 24 hours. The periods ranged from 21 hrs. 45 min. to 24 hrs. 20 min. with 75% between 23-24 hrs.

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THE EFFECT OF PROLONGED ORBITAL FLIGHT ON THE CIRCADIAN RHYTHMS OF POCKET MICE

EXPERIMENT DEFINITION

Background

The first formal record of the existence of biological rhythms is probably by the astronomer De Mairan who in 1729 described diurnally periodic leaf movements in plants held in the dark. Since that time the literature has become repleat with evidence of diurnal periodicity in many forms of life at many levels or organization. So universal is the occurrence of this rhythm that it is generally excepted as an inherent property of life. Present day research emphasizes on one hand the mechanism of the so called "biological clock" and on the other the coupling of the clock to environmental stimuli.

It is pertinent to emphasize that few precise 24-hour rhythms have been documented. Variations in period of 22-26 hours may occur depending upon the species studied. A variation of 1-2 hours is not unusual between individuals of the same species and a few experiments have been done involving crossing strains of different rhythms. As a result, the commonly used term for the phenomenon of diurnal periodicity is "circadian" rhythm, meaning literally "about a day".

It is also pertinent to note that many individual rhythms, usually not in phase, within a single organism can be measured and in some way probably integrate to form the dominating circadian rhythm of the total organism. Considerable emphasis is being placed on study of these subrhythms using the Fourier Analysis as the statistical tool.

Whether the workings of the clock is predominantly a chemical, physical, biochemical, or biophysical phenomenon is argumentative on the basis of existing data. The study of biorhythms in space theoretically would permit some resolution of the question as to whether terrestrial stimuli indeed set the period or simply change its phase. The purists generally feel that a satellite in orbit 200-300 miles above the earth's surface may still be within the influence of "pervasive geophysical forces" which may be sensed by biological material. Therefore, a heliocentric orbit would be highly desireable for this kind of study. A circumlunar orbit would be a compromise since the possibility exists of periodically approaching the earth at a frequency which would entrain biological rhythms and mask the effects being looked for.

There is yet to be studied a physical or environmental stimuli to which some form of life does not react. It is not improbable that man as a product of 3-1/2 billion years of terrestrial evolution senses stimuli of which he is not aware. If the circadian rhythms of man are in any way coupled with terrestrial cues the probability of his satisfactory performance on prolonged space missions will be low. Within this context studies of circadian rhythms in a variety of life forms placed in deep space can have as high a priority as the required measurement of ionizing radiation in space.

Data from metabolic studies on pocket mice show that Perognathus longimembris has a circadian metabolic rhythm which can be detected at both moderate (22-24°C) and low (10°C) environmental temperatures, at high and low humidities, in the dark or under normal photoperiod, with and without food, in normal atmospheres and 100% oxygen, and in both individually housed and in grouped mice. It is anticipated that placing these animals in earth orbit will elucidate the effects of exogenous factors which may influence a persistent endogenous rhythm. While the most obvious exogenous cues to be studied are weightlessness and orbital period, the experimental design is easily adaptable to provide for the input of almost any specific environmental stimuli in the isolation of space.

Experimental Design

Dr. Colin S. Pittendrigh of Princeton University and Dr. R. G. Lindberg of Northrop Space Laboratories have proposed that six Perognathus be flown in conditions of constant darkness and temperature at about 22°C for a minimum of 21 days. Their body temperature and locomotory activity* will be recorded; the former by intraperitoneally implanted temperature transmitters and the latter either by AM modulation of the transmitter signal by magnetometer or capacitor "gates" along the length of the container.

The data will be subjected to frequency spectrum analysis, which shows for grounded animals a clear circadian component. The question being asked is, Does the frequency spectrum for either parameter (temperature or locomotion) change when all geophysical variables (other than light and temperature) are either removed or are sensed by the animals with a period of about 90 minutes instead of 24 hours?

The hypothesis predicts:

1. There will be no loss of the circadian component in the frequency spectrum; and
2. There will be no new component introduced by an orbital period of about 90 minutes.

The six animals flown will have been ground tested for their capacity to withstand launch and pre-orbital flight conditions. They will also have had their individual frequency spectra fully measured for one month before flight.

* Measurement of animal activity is completely feasible; however, provision for such measurements does increase the complexity of the experimental hardware. Until there is either a better scientific justification than presently available or a significant relaxation of spacecraft constraints occurs, only body temperature data will be collected.

The experiment design is adaptable to earth orbit, as lunar or heliocentric orbits provided that a telemetry link is available negating the requirement to recover the experiment package.

The Experimental Animal

Perognathus is almost uniquely suited for this experiment. It has an unusually large (3-5°C) amplitude to its daily temperature cycle; weighs 8-10 grams and, most significantly, it has no water demands. Urine production is extremely low (0.05 ml/24 hrs) and the feces are dry and odorless (0.1 gram/day). The problems of watering and waste removal for mammals in zero-g are therefore either completely eliminated or easily solved.

Experiment Package Concept

Each mouse will be individually housed in a thin wall aluminum cylinder twelve to eighteen inches long and one and one-half inches in diameter containing 30 grams of loose sun flower seeds (30-day supply). An oxygen nitrogen atmosphere at 14.7 psi, or an oxygen atmosphere at 5 psi, will be provided either as a part of the experiment package or from the space vehicle environmental control system. The atmosphere will flow through the tube at a rate of ~ 0.03 cfm, at a constant temperature (~ 75°F), and constant relative humidity (~ 50%). At the exhaust end of the tube, coarse screening will prevent the seeds from being blown from the tube under zero-g conditions, but will permit the escape of feces (.05-0.1 gram/24 hours) and seed particles into a debris trap. The lining of the tube will consist of a fine mesh screen impregnated with an absorbant asbestos-cellulose material. The lining will serve three purposes: (1) absorption of urine (0.05 ml/24 hrs), (2) foot hold for the mice, and (3) shielding for electronic instrumentation.

Three kinds of sensors can be provided. Ambient temperature will be monitored by a thermistor in the exhaust air of each tube. Body temperature will be monitored by an implanted telemeter device and a loop antenna running the length of the cylinder. Locomotory activity can be monitored. The thermistor, antenna, and motor activity leads, if present, will feed into an electronic instrumentation package which in turn will feed into a data storage device and on demand into the satellite telemetry system.

It is anticipated that under zero-g conditions, the steady, near laminar flow of air through the relatively smooth cylinder will continually carry the loose seeds and debris to the exhaust end of the tube irrespective of the animal's behavior.

Six individual cylinders will be grouped in a geometry most compatible for inclusion in a specified spacecraft. It is not essential that the individual cylinders be close together or that the data instrumentation package be located immediately adjacent to the tubes.

A graphic concept of the minimum experiment package for monitoring both temperature and activity (i.e., without data handling and environmental control system) is shown in Figure 1. The six tubes are assembled in two layers of three tubes each. The instrumentation packages are mounted on a common panel adjacent to the tubes. A constant quality atmosphere from the spacecraft ECS enters the assembly through a manifold and is exhausted through a manifold at the opposite end. Mounting brackets will be provided at each end of the assembly to match the mounting requirements of the spacecraft. A graphic concept of a "self contained" package for a 30-day mission is shown in Figure 2 in which data handling and storage, and an environmental control system is provided. In this concept locomotory activity is not monitored. The package is estimated to weigh less than 25 pounds, occupy less than 1 cu. ft. of space and utilize 1-3 watts of power.

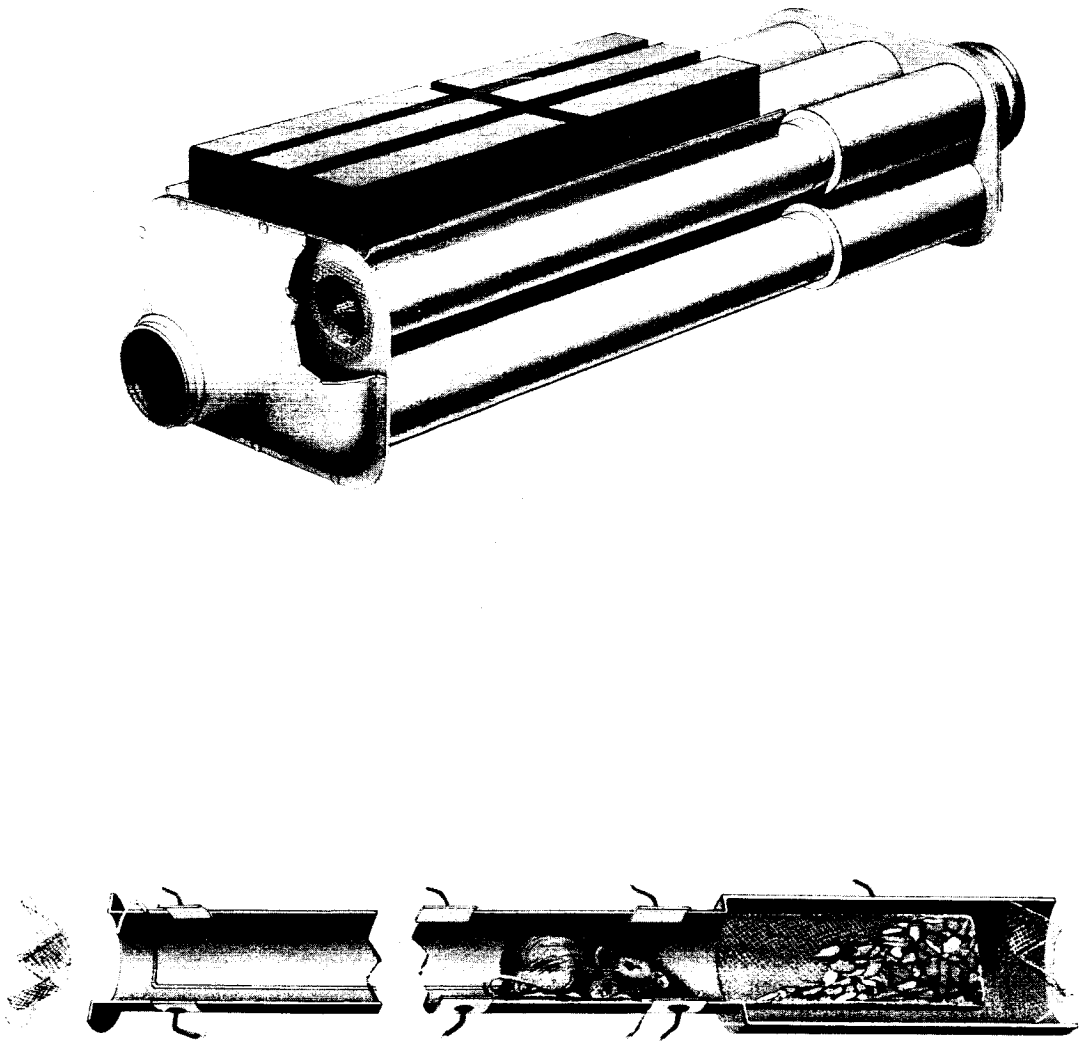

FIGURE 1 CONCEPT OF AN EXPERIMENT PACKAGE TO STUDY CIRCADIAN RHYTHM IN POCKET MICE DURING PROLONGED ORBITAL FLIGHT

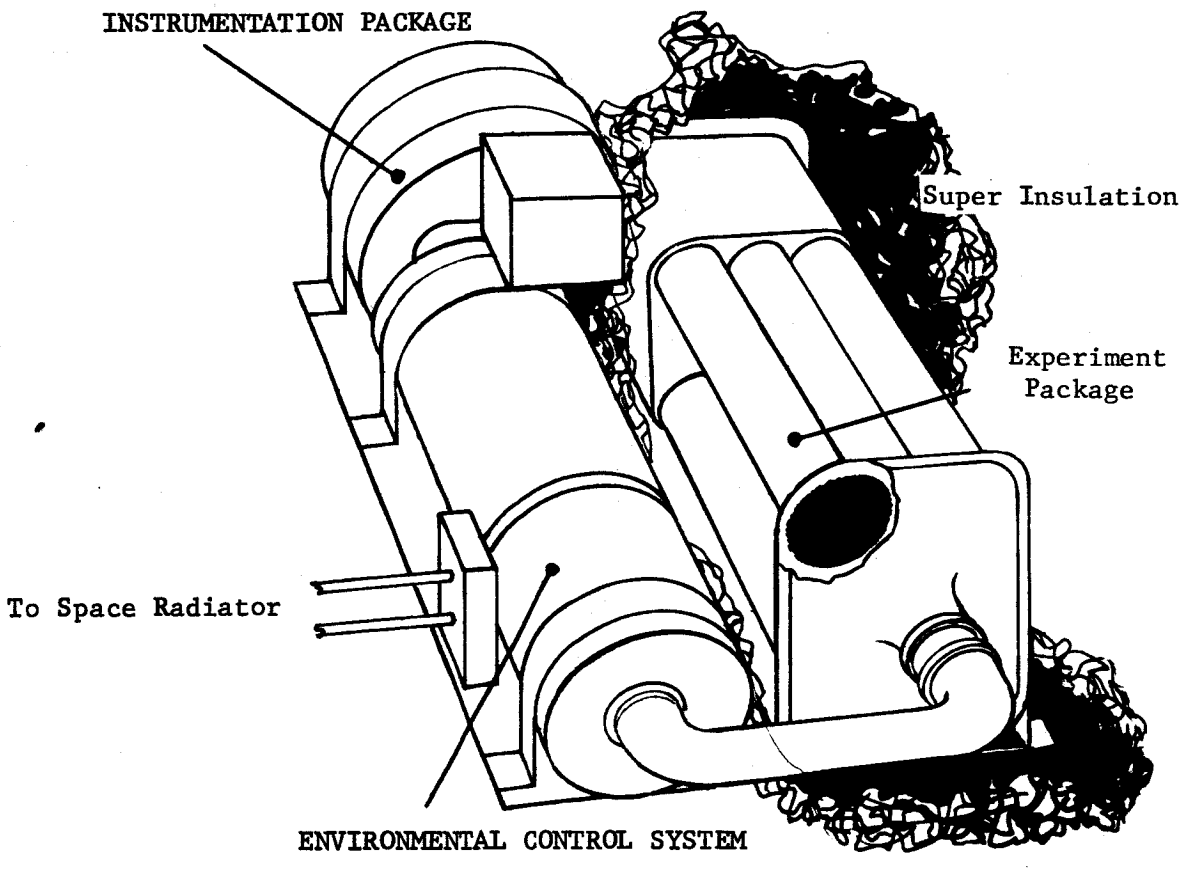

FIGURE 2 CONCEPT OF AN EXPERIMENT PACKAGE TO STUDY CIRCADIAN RHYTHM IN POCKET MICE DURING PROLONGED ORBITAL FLIGHT

LIFE SUPPORT HARDWARE

Animal Housing and Feeding

Each mouse will be individually housed in a thin walled metal cylinder with a wire mesh liner coated with an absorbent material such as asbestos, long fibered cellulose, or fibrous carbon. The purpose of this liner is to provide footing for the animal, absorption of urine, grooming, and R.F. shielding.

Laboratory tests were conducted to determine the effect of long confinement in tubes on pocket mouse behavior and well-being, and to test the effectiveness of the liner materials. one and one-quarter inch diameter screen tubes of various lengths inserted into either metal or plastic sleeves were used in the study.

There is good evidence to show a suppression of the hypometabolic periodicity in tubes 9 inches in length whereas the periodicity appeared normal in tubes 15 inches in length (see Section II). The optimum size tube is estimated to be between 12 and 15 inches in length and 1-1/2 inches I.D.

Tests with fiberglass, aluminum, copper and stainless steel in mesh 16 x 16 to 10 x 10 revealed that .025 inch diameter stainless steel wire cloth (10 x 10 mesh per inch) was most resistent to gnawing and most compatible with application of the absorbents.

Asbestos was a good absorbent and an excellent substrate for grooming activities. However, it could be scratched from the screen tube in sufficient quantity to create a problem in a closed circuit filtering system. Long fiber cellulose is also scratched from the screening but in considerably less quantity with the major portion of the loosened fibers tending to remain attached and projecting into the tubes somewhat like velvet. Urine is absorbed particularly well with this arrangement. The

The grooming characteristics are acceptable, but somewhat less than asbestos. Charcoal fibers have also been suggested, but as yet have not been tested.

Food was provided loose in the tubes as proposed for a space package. Husked sunflower seeds were consumed at an average rate of 0.8 grams/day/mouse with a minimum of residue. Thus, 30 grams of husked seeds will provide a 25% margin of safety over the 24 grams calculated to be required for a 30-day study.

Environmental Control System

Consideration of an environmental control system for the proposed experiment was not a contractual commitment. The information presented here represents preliminary planning with the specific purpose of establishing, (1) the feasibility of a "self contained" experiment, and (2) approximate weight, volume and power requirements. Preliminary analysis suggested that a relatively passive ECS of high reliability could be achieved using metallic superoxides as the principle active material. It is recognized that such a system is unproven in terms of the precision of regulation called for in experimental biology, but its apparent simplicity cannot be denied. More conventional stored gas systems utilizing either 100% oxygen at 5 psi or oxygen/nitrogen at 14.7 psi were also considered and in the final analysis may be the preferred choice, depending upon the constraints placed on the experiment hardware by any particular spacecraft.

ECS Requirements

Temperature	75 ± 2°F
Oxygen Required	4-100 ml/hr/mouse
Water Production (Respiratory)	4-100 mg/hr/mouse
Urine	1 ml/24 hrs/mouse
RQ	0.75
Pressure	14.7 psi
Atmospheric O_2	82-25%
Atmospheric N_2	82-75%
Atmospheric CO_2	3%
Relative Humidity	40-50% at 75°F
Air Flow	0.03 CFM

Environmental Control System Concept

The Environmental Control System (ECS) shown in Figure 3 is designed to provide a life supporting atmosphere for the mice and maintain atmospheric and electronic temperatures within the required tolerances. An initial 20% oxygen 80% nitrogen atmosphere is maintained by a demand type metallic superoxide system. This system incorporates either KO_2 or NaO_2 as the principle reactants and is complemented as required by activated charcoal and LiOH. Carbon dioxide and water generated by the animals is adsorbed by the superoxide to produce O_2 according to the following basic exothermic chemical reactions.

$$2\ KO_2 + H_2O \rightarrow 2\ MOH + 3/2\ O_2$$

$$2\ KOH + CO_2 \rightarrow K_2CO_3 + H_2O$$

$$2\ KOH + 2\ CO_2 \rightarrow 2\ KHCO_3$$

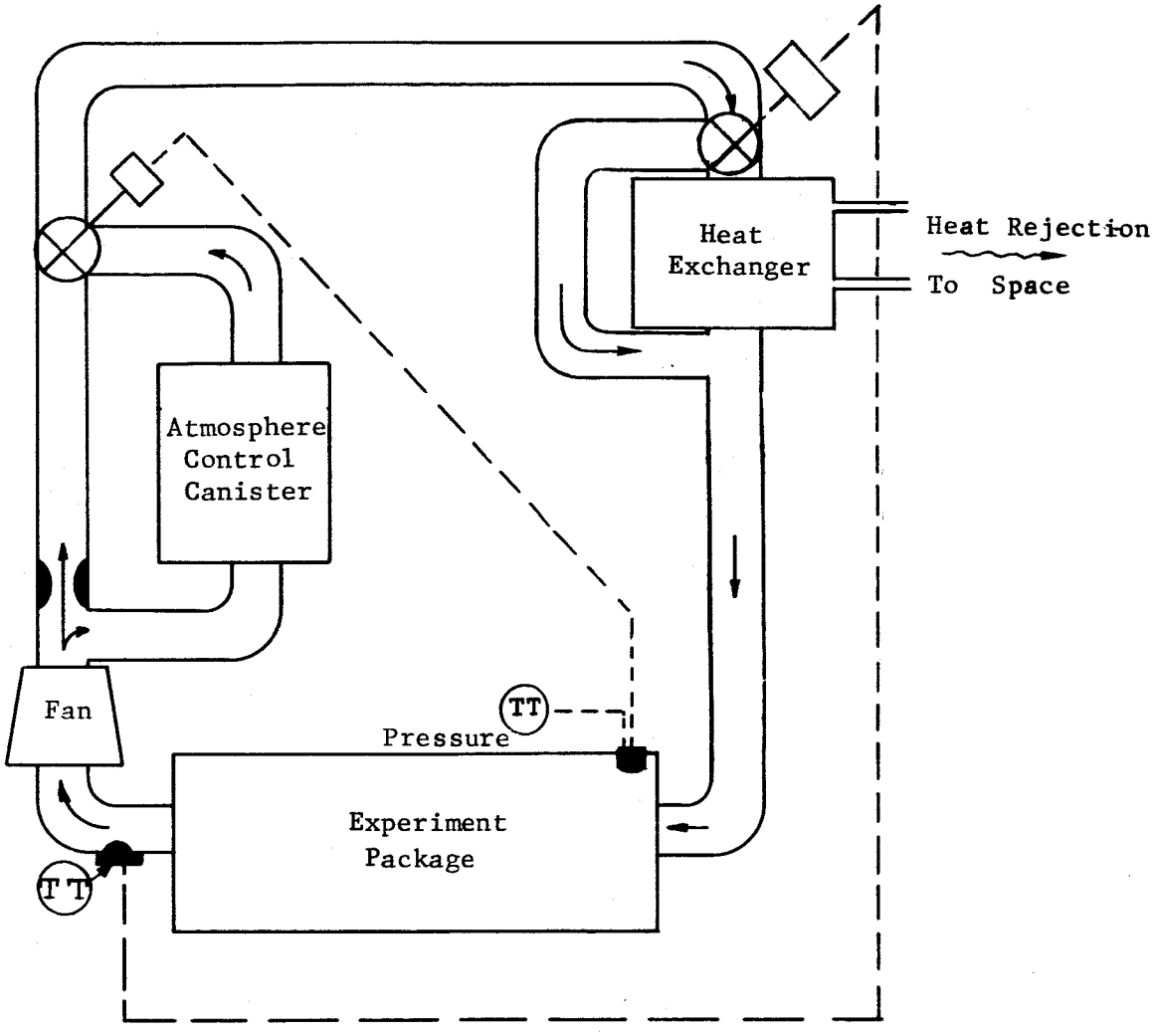

FIGURE 3 ENVIRONMENTAL CONTROL SYSTEM FOR POCKET MICE EXPERIMENT

Carbon fibers are available which have been made by MSAR which have 150% the absorptive capacity of the CCL_4 standard. The fibers can be formed to various shapes and densities. Absorption of water vapor is excellent at relative humidities greater than 50%. The use of a carbon fiber bed around each animal tube would serve the dual purpose of dehydration of the atmosphere and adsorption of volatile organic materials from urine and feces.

Thermal Control

The temperature control for the experiment package has been designed such that the entire experiment package is "self contained". The entire package except for the radiator is insulated from its external environment by wrapping in multiple layer foils of aluminized mylar super insulation. This minimizes the effects of the unknown external environment on temperature control. The temperature control maintains the temperature of the experiment package at $75 \pm 2°F$, and also maintains the temperature of the electric and electronic equipment at $100 \pm 25°F$. Since the entire package, except for an external radiator, is insulated from its external environment, the primary part of the heat load is made up of the metabolic heat, the heat of absorption in the lithium hydroxide cannister chemical heat of reaction, and the electrical and electronic heat of the equipment. Preliminary analysis indicates that this heat load may be dissipated by an external radiator which conductively cools a heat exchanger in the Gas Loop of the ECS. Temperature is regulated by control of the air flow through this heat exchanger. An alternate approach which could be adopted is to provide a secondary glycol circuit to provide the heat transport from the sources of heat to the terminal point of heat rejection. In both cases a radiator provides the necessary heat rejection from the package. The size of radiator required to dissipate the heat is a function of the position of this radiator. If the radiator is positioned such that it is normal to the solar rays, a radiator of 4 ft^2 will be required. If the radiator is positioned such that it is never irradiated by the sun, the radiator size

required is approximately 0.5 ft^2. If locating a radiator on a spacecraft becomes impossible, heat rejection can be provided by a water boiler. However, the weight of water required for a 30-day mission is in excess of 75 pounds which is seven times greater than the dry ECS weight.

DATA HANDLING SYSTEM

The main function of the data handling system for this proposed experiment is the measurement of temperature from each animal. This data is accurately converted into a convenient format, accumulated in a buffer storage device, and retrieved and transmitted to the ground station on command via the spacecraft telemetry system.

The simplified block diagram of Figure 4 shows the experiment instrumentation for the temperature and housekeeping data subsystems. Also indicated are the associated data processor (one channel shown), the programmer which controls all data channels (including housekeeping measurements), and the data storage system.

To achieve the experiment objectives, pocket mouse temperature measurements must be made with a resolution of 0.1°C. This temperature resolution must be preserved, together with its corresponding time base accuracy, through the chain of events up to and including ground data reduction. The practical implementation of a system meeting these requirements is best provided by using digital techniques. Studies under contract NASw-812 have led to the design of a digital system capable of fulfilling the above requirements. The following paragraphs discuss the major elements of this system.

Temperature Telemeter

Temperature measurements are derived from a thermal sensitive telemeter implanted in the animal which accurately transmitts mouse body temperature to within 0.05°C. An antenna is used to detect the telemeter pulsed RF energy and a receiver converts the signal into a series of pulses which are periodically sampled by the data processor. These telemeters have

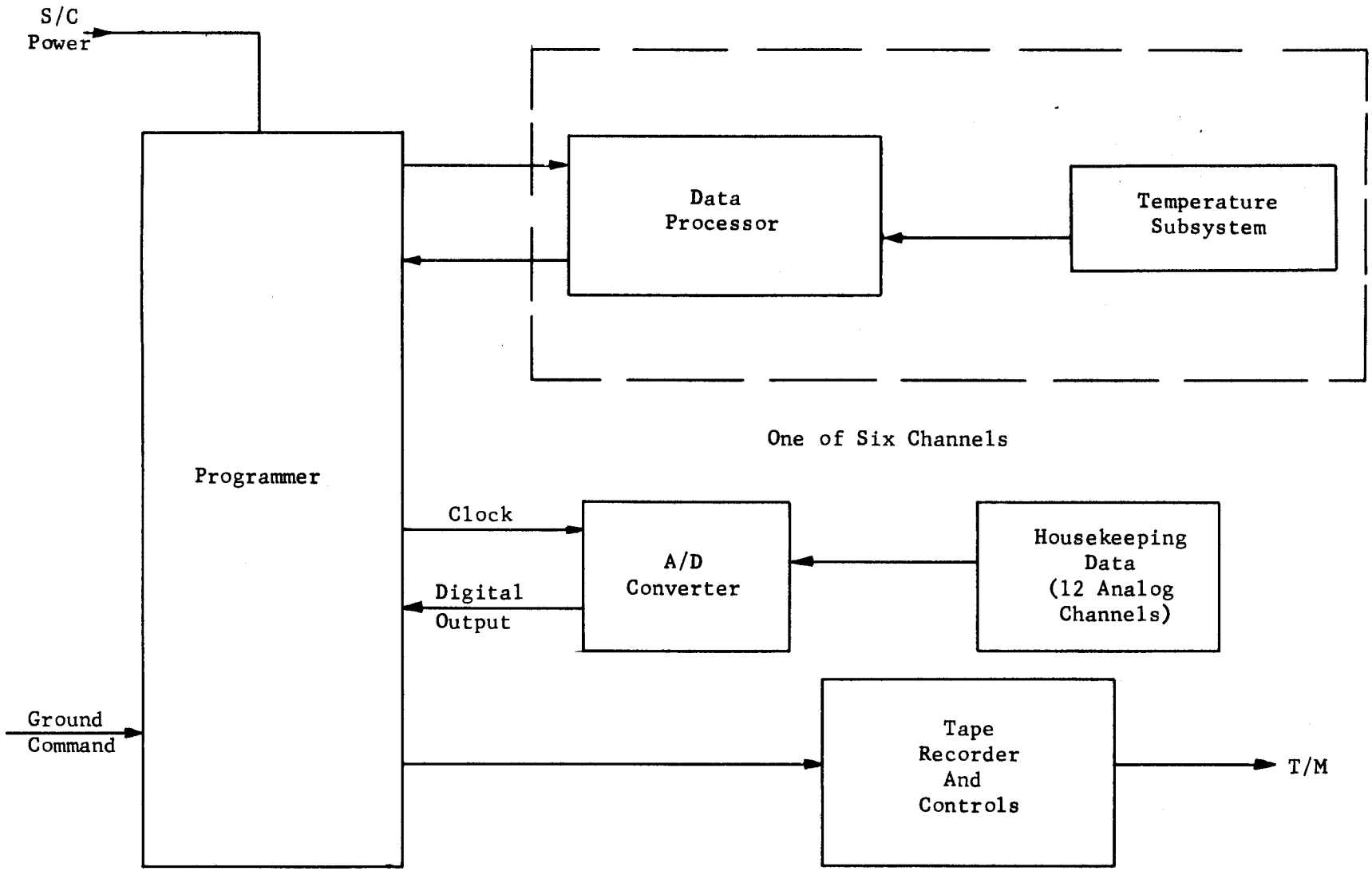

FIGURE 4 SIMPLIFIED BLOCK DIAGRAM OF INSTRUMENTATION

been successfully used at NSL and Princeton University with pocket mice to generate data of the type and quality required for the orbital experiment.

Data Processor

Temperature pulses from each of the six channels are fed into the data processor and converted into 10-bit digital format prior to storage. The data processor basically consists of a 10-bit binary counter, a 10-bit shift register and the required logic elements to organize the temperature data into the required format. Pulses from the temperature subsystem are gated into the data processor by 10 second timing pulses from the programmer where timing accuracies are maintained to 0.01 per cent.

At a nominal temperature, the telemeter pulse rate is approximately 350 pps and produces a total of 3500 counts in 10 seconds. The capacity of the 10-bit binary counter in the data processor is $(2^{10}-1)$ or 1023. However, the counter recycles every 1024 counts and at the end of 10 seconds has recycled three times and the registered count will be in the fourth cycle. In effect, this constitutes a 12-bit digital system where the first two significant bits (or first 3072 counts) are not recorded since they are always known. The resolution of the system is about 0.025°C providing a total ambiguity of 0.05°C.

Programmer

The data processor and data storage tape recorder are controlled by the programmer subsystem. The programmer sequentially selects each channel at the proper time and shifts its data into the tape recorder. A graphic presentation of work and frame sequences is shown in Figure 5. The main elements of the programmer and the functions of these elements are as follows:

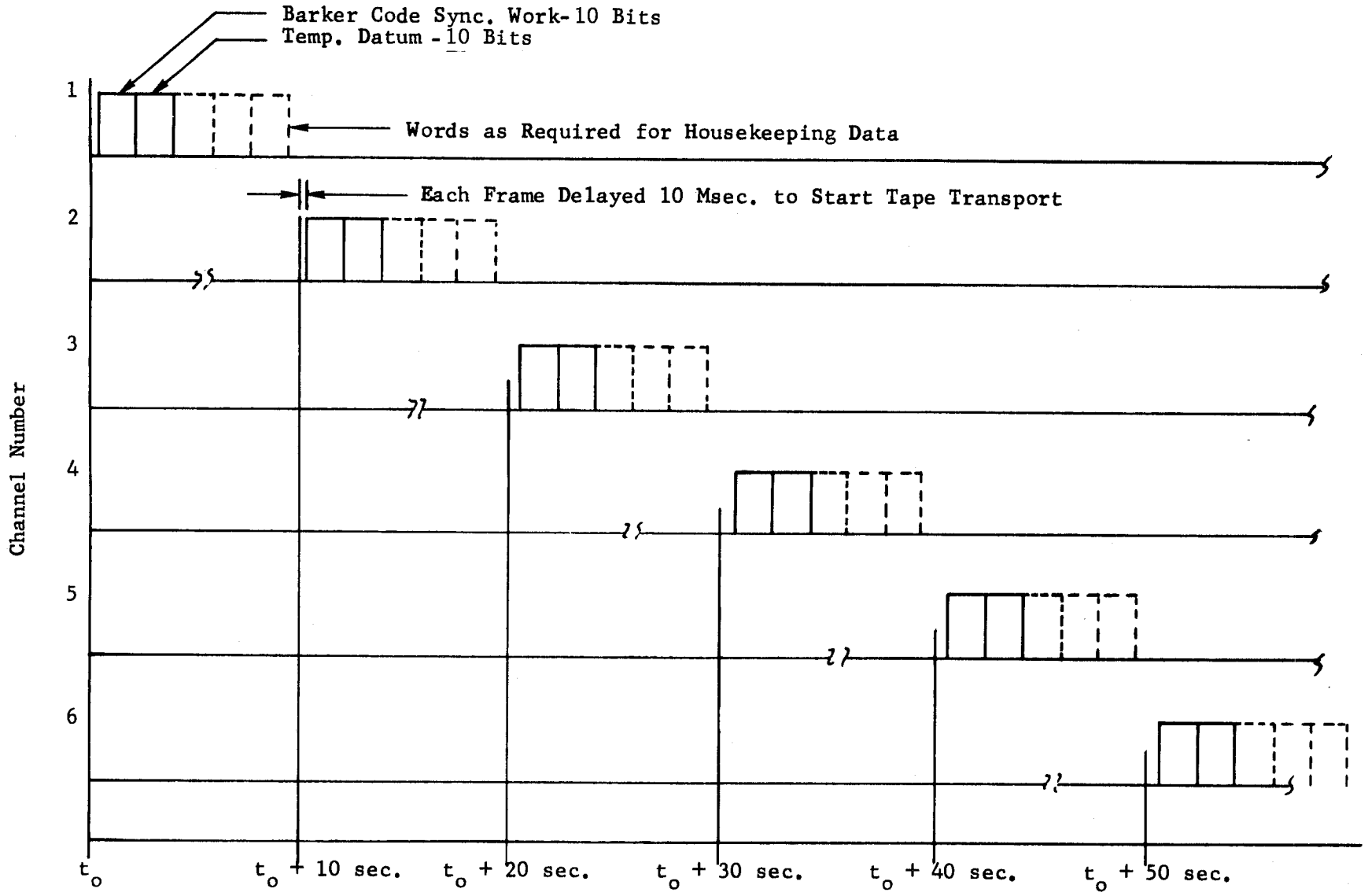

FIGURE 5 EVENTS PER FIRST 50 SECONDS OF EACH 5 MINUTE CYCLE

a. Clock - A 10 KC crystal control oscillator is divided down to produce pulses at several lower frequencies. These pulse trains are used for the timing and synchronization of the events controlled by the programmer.

b. Channel Selector - Each stage of a six-stage ring counter sequentially enables a set of three gates. Each of these sets of gates control the operations required to shift data out of a particular data processor and reset it to accept the next temperature data point.

c. Shift Pulse Generator - This circuit is a four-stage binary counter pre-set to the count of 10. Upon the receipt of a re-set pulse, it generates a train of 10 shift pulses at the clock rate of 500 pps. A group of these pulses are generated for each to be shifted out of the data processor's shift register and into storage.

d. Word Selector - The word selector is a ring counter with a stage for each word in the data frame. It generates pulses to recycle the shift pulse generator. It also produces control pulses for re-setting the particular data processor being interrogated.

e. Recorder Control - The data storage tape recorder is turned on 10 msec prior to the recording of the first word in each frame to allow the tape transport to come up to operating speed. The control curcuit is a flip-flop that drives the control circuits in the tape transport. This flip-flop is turned off at the end of the last word in each frame.

f. Barker Code Sync Generator - A digital system requires a synchronizing word at the beginning of each frame in order to synchronize data processing systems with the recorded data. The Barker Code also establishes bit synchronization.

g. Housekeeping Data - To record housekeeping measurements, an analog to digital converter is used in conjunction with the programmer. It digitizes the several analog voltage from the temperature and pressure transducers and from electronics circuits being monitored.

h. Recorder and Controls - The biorhythms experiment dictates the use of data storage because data is taken each five minutes and is transmitted to ground stations only once per satellite orbit. Cost considerations, complexity, and flexibility dictate the digital tape recorder as the logical choice for this data storage function. The digital data is stored in serial form on one track of a two track recorder. The second track records clock pulses to insure bit synchronization during data reduction. A quarter inch or half inch tape will be used in the form of a continuous loop about 36 inches in length. A single head for each track will serve for both record and playback. No erase head is required since the record head always saturates the tape during the record mode. Two control systems are required; one to start and stop transport in the record mode, and one to playback on command. On command to playback, the transport advances the tape in the record mode to the end of the loop (at splice). This erases any bits left on the remaining tape at the end of the last orbit. When the end of the loop is reached, the recorder automatically switches to playback mode and the tape continues to advance feeding "non-return to zero" PCM data to the spacecraft telemetry system. The tape recorder continues to operate in playback mode through several cycles to insure a good transmission and ground station recording. The time required for one playback of the data taken during one orbit is approximately 10 seconds. On command the recorder switches back to the record mode the next time it reaches the starting point on the tape loop. The tape speed for both record and playback is approximately three inches

per second and the packing density is approximately 200 bits per inch. Tape speed is not critical since a time reference is used on the adjacent track.